

POLARIZED LIGHT SPECTROSCOPY AND MOLECULAR ORIENTATION

Lennart B. Å. Johansson



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POLARIZED LIGHT SPECTROSCOPY AND MOLECULAR ORIENTATION

BY

LENNART B.-Å. JOHANSSON

DEPARTMENT OF PHYSICAL CHEMISTRY 2

CHEMICAL CENTRE, P.O.B. 740

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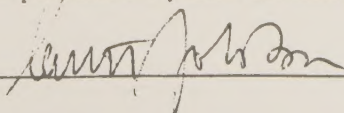


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Abstract Methods based on polarized light spectroscopy especially adopted to the study of <u>molecular orientation in biological membranes</u> are presented. A general theoretical treatment is given on the absorption and emission of chromophores in anisotropic systems. From <u>linear dichroism (LD)</u> studies of chromophores in macroscopically aligned lipid bilayers the molecular orientation in terms of order parameters is yielded. This information may also be obtained by fluorescence detected linear dichroism (FDLD) which is a method developed here. A prerequisite in all orientational studies utilizing polarized light spectroscopy is the knowledge of the directions of the <u>electronic absorption and emission transition moments</u> in the chromophores. These polarizations in the isoalloxazine ring were determined. The orientation of β-carotene, all-trans-retinal and some local anesthetics in model membranes have been investigated with LD. The phase structure of two <u>liquid crystalline phases</u> that spontaneously orient in a magnetic field were investigated with ^2H NMR and LD. It is concluded that both phases consist of long <u>rodlike aggregates</u>. The liquid crystals are suitable as <u>ordering matrices</u> in light spectroscopy. The use of time resolved fluorescence in orientation studies is briefly discussed. The orderparameters in <u>macroscopically isotropic systems</u> such as vesicles, micelles or cubic phases could then be obtained.			
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Polarized Light Spectroscopy and Molecular Orientation

This thesis is based on the following publications, which are referred to by Roman numerals in the text.

- I. Orientation and Mobility of Molecules in Membranes Studied by Polarized Light Spectroscopy
L.B.-Å. Johansson and G. Lindblom, Q.Rev.Biophys. 13, 63 (1980).
- II. Linear Dichroism as a Tool for Studying Molecular Orientation in Membrane Systems. 2. Order Parameters of Guest Molecules from Linear Dichroism and Nuclear Magnetic Resonance
L.B.-Å. Johansson, Å. Davidsson, G. Lindblom and B. Nordén, J.Phys.Chem. 82, 2604 (1978).
- III. Electronic Transitions in the Isoalloxazine Ring and Orientation of Flavins in Model Membranes Studied by Polarized Light Spectroscopy
L.B.-Å. Johansson, Å. Davidsson, G. Lindblom and K.R. Naqvi, Biochemistry 18, 4249 (1979).
- IV. Orientation of β -Carotene and Retinal in Lipid Bilayers
L.B.-Å. Johansson, G. Lindblom, Å. Wieslander and G. Arvidson, FEBS Lett., in Press.
- V. Studies of Chromophores in Model Membranes by Polarized Light Absorption Spectroscopy. The Orientation and Binding of Tetracaine and Procaine
L.B.-Å. Johansson and G. Lindblom, Biophys. J., in Press.
- VI. The Structure of a Lyotropic Liquid Crystalline Phase that Orients in a Magnetic Field
O. Söderman, G. Lindblom, L.B.-Å. Johansson and K. Fontell, Mol.Cryst.Liq.Cryst. 59, 121 (1980).

VII. A Magnetic Field Orientable Lyotropic Liquid Crystal.

An Ordering Matrix in Light Spectroscopy.

L.B.-Å. Johansson, O. Söderman, K. Fontell and G. Lindblom,
submitted.

VIII. Fluorescence Detected Linear Dichroism. A New Method for
Studies of Molecular Orientation in Uniaxial Systems.

L.B.-Å. Johansson, G. Lindblom and K.R. Naqvi, J. Chem. Phys.,
in Press.

1. INTRODUCTION

Polarized light spectroscopy has long been utilized in studies of molecular orientation. The absorption or emission of polarized light of a macroscopically aligned sample containing chromophores is then investigated. The orientation can be obtained since the absorption and emission of linearly polarized light occur along well-defined directions in the molecules.

Methods based on polarized light spectroscopy especially adapted to the study of molecular orientation in biological membranes are presented here. The chromophores in membranes are mainly located in the proteins as tyrosine, tryptophan, hemes, flavins etc. Others present are for example carotenoids, ubiquinones and medical substances. These molecules are all present in very small amounts and in order to study them sensitive and selective methods are needed. Light spectroscopic methods are then very suitable since they are extremely sensitive, by several orders of magnitude compared to NMR (1) for example. NMR (2-5) and X-ray (6) techniques have however successfully been used in the investigations of membrane lipids.

In the present thesis I-III deal with a method for studying the molecular orientation in macroscopically aligned lipid bilayers. Emphasis is made on the experimental precautions and the characteristics of the chromophores that are necessary in order to get relevant information. A general theoretical treatment is also given on the absorption and emission of chromophores in anisotropic systems. The orientation of some important biological chromophores in model membranes is presented in IV and V.

Two liquid crystalline phases that spontaneously orient in a magnetic field are investigated in VI and VII. From the polarized absorption and NMR results obtained conclusions about the phase structure has been drawn.

In the last paper, VIII, a method to study the polarized absorption via the fluorescence phenomenon is introduced. The strength of this fluorescence detected linear dichroism method (FDLD) is the possibility to study the orientation in samples having very low absorbance. In the final section of this thesis (4.2) I will briefly discuss the possibilities of using time resolved fluorescence in membrane studies.

1.1 Liquid Crystalline Phases

An amphiphilic molecule contains two parts differing in water solubility. The water repelling, hydrophobic part, consists usually of one or several hydrocarbon chains attached to the water attracting, hydrophilic part. Typical examples are a phospholipid, as dipalmitoyl lecithin and a detergent, as potassium dodecylsulphate (cf. Figure 1.1).

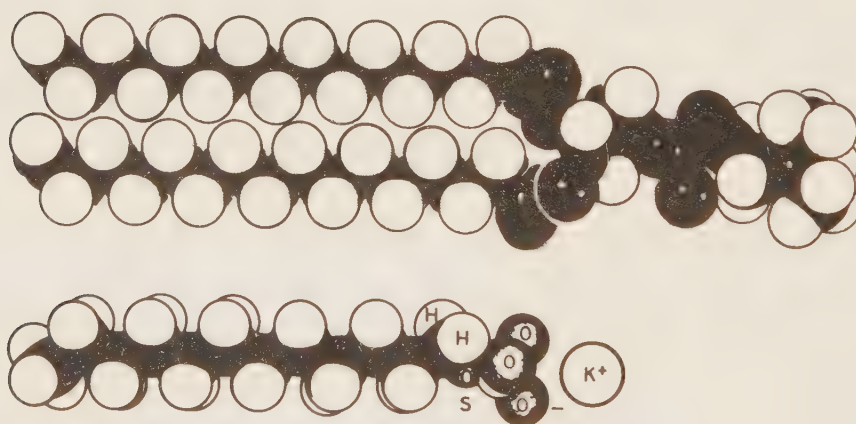


Figure 1.1. Space filling molecular models of a: dipalmitoylphosphatidylcholine, b: potassium dodecylsulphate.

Amphiphiles and water form different phases depending on the concentration. The phases are isotropic solutions of micelles or liquid crystalline structures that consists of spherical, rodlike or lamellar aggregates. These lyotropic liquid crystals have properties intermediate between the ordered crystalline state and the liquid state. The liquid crystalline phases are characterized by a low short range order of the amphiphilic hydrocarbon chains reminding of the liquid state. There exist, however also a long range "crystal-

line" order in one, two or three dimensions. Structurally most of the liquid crystalline phases are built up from lamellar or rodlike aggregates. In the latter structures long rods may be stacked together in an one dimensional array or cross-linked to form two and three dimensional networks. The lamellar structures consist of large coherent amphiphilic lamellae separated by water regions.

Amphiphiles form depending on composition and temperature structures of various kind. Figure 1.2 shows the phase diagram of monoolein and water together with the different liquid crystalline structures.

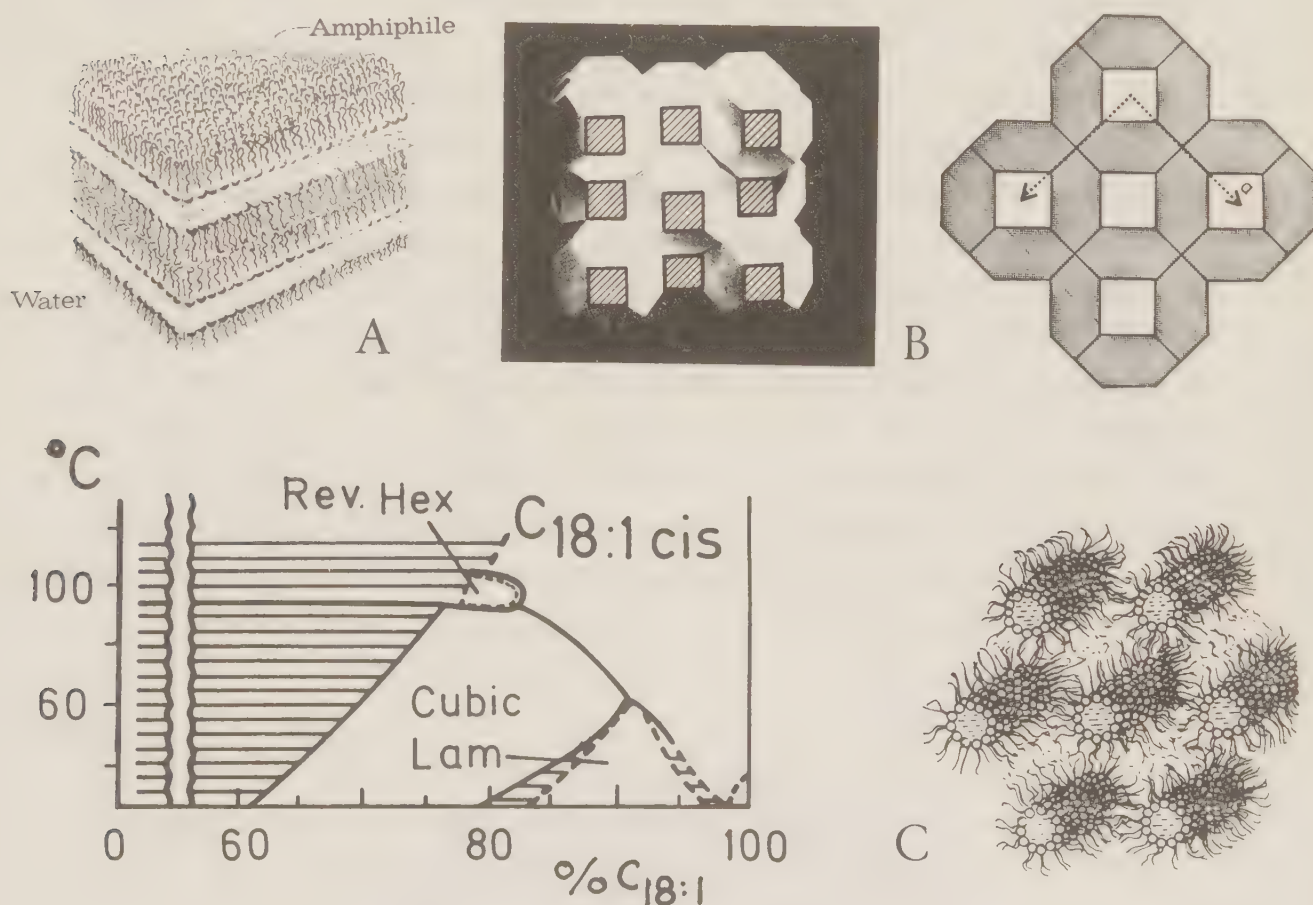


Figure 1.2. The binary phase diagram of monoolein and water. Schematic pictures of A: the lamellar, B: the cubic and C: the reversed hexagonal phases. In the cubic phase the hexagon-shaped surfaces represent the lipid bilayer units and the square-shaped areas the water channels.

Recently theoretical models predicting the appearance of phase diagrams have been published (7,8). In the theory by Israelachvili et al. (8) the geometry of the amphiphile plays an important role in determining the aggregate shape. Such geometrical considerations have also satisfactorily explained the lipid compositions found in the bacterial membranes of Acholeplasma laidlawii (9).

1.2. Biological Membranes

Every living cell is enclosed by a membrane that serves not only as a cavity inside which the cell can function but also as a discriminating gate, through which essential substances can enter and waste products can leave. The dominating components of the membranes are lipids and proteins which vary depending on the type of cell. Common lipid constituents are glycerolipids, which are in majority, further sphingolipids, sterols and carotenoids. The glycerolipids usually form lamellar structures and according to a general membrane model proposed by Singer and Nicholson (10) these lamellae serve as matrices for the various proteins. This model called the fluid mosaic model is displayed in Figure 1.3. Other liquid crystalline structures may also be of importance in biological membranes systems (11-13). The plastids in etiolated Avena leaves contain prolamellar bodies (14) having a structure very similar to the cubic phase (cf. Figure 1.2) found in the mono-olein-water system (15,16). Non-lamellar phases are probably also involved in the fusion processes in living cells (12,13,17).

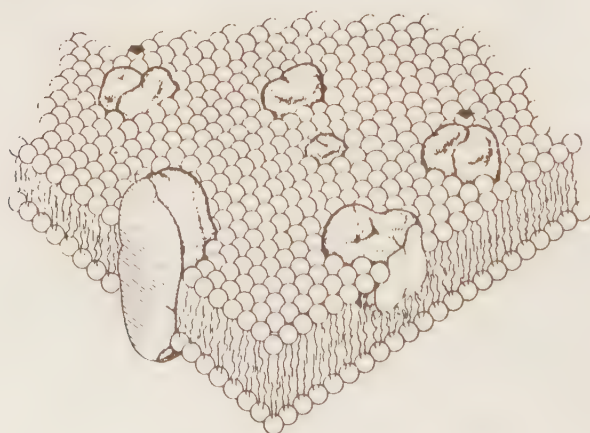


Figure 1.3. A schematic representation of a biological membrane.

2. LINEAR DICHROISM IN MEMBRANE STUDIES

2.1. The Linear Dichroism Method

The absorption of light in the wavelength region 100-1000 nm is usually associated with transitions between different electronic-vibrational states. For all but some very weak transitions the absorption is extremely well described by the electric dipole approximation (18). Then the molar absorptivity or the absorbance (A) is related to the electric transition dipole moment, $\vec{p}$, and the polarization of light $\vec{E}$, through

$$A = k(\vec{p} \cdot \vec{E})^2 = k|\vec{p}|^2|\vec{E}|^2 \cos^2\theta \quad (2.1)$$

where k is a constant (19). The absorbance clearly depends on the angle (θ) between the transition dipole and the electric field vector of light. Therefore in macroscopically anisotropic systems the absorbance is also anisotropic (cf. I). The differential absorption of such a system of two, mutually perpendicular, linear polarizations is called the linear dichroism i.e. $LD = A_{||} - A_{\perp}$. The simplest experimental equipment necessary for linear dichroism studies is an absorption spectrophotometer and a pair of linear polarizers. This method of separately measuring $A_{||}$ and $A_{\perp}$ is for many purposes excellent but becomes imprecise for small dichroisms. A more accurate and up-to-date method is offered by the light modulation techniques. A variety of techniques are available but all of them have one component in common namely a device to modulate the state of polarization. This device may be a rotating polarizer (20) or more often a plate whose birefringence is periodically variable. In photoelastic modulators an isotropic transparent material, (silica or CaF_2) is periodically compressed with a frequency ω so that a birefringence, $\Delta n = \Delta n_0 \sin \omega t$, is induced (21). The polarization of a linear polarized beam, impinging perpendicular to the induced optical axis, can be resolved into one component parallel and another one perpendicular to this axis. The phase shift $\delta = \delta_0 \sin \omega t$, caused by the birefringence,

will provide a transmitted beam whose polarization state is time dependent. By passing the phase modulated light through a dichroic sample different polarizations will be unequally absorbed. The analytical expression of the transmitted intensity, $I(t)$, calculated by using the convenient Stokes-Müller formalism (22, 23) is

$$I(t) = I_0 10^{-\bar{A}} \left\{ \cosh\left(\frac{LD \ln 10}{2}\right) + \sinh\left(\frac{LD \ln 10}{2}\right) \cos(\delta_0 \sin \omega t) \right\} \quad (2.2)$$

where $\bar{A}$ is the average absorbance and I_0 is the intensity of the incident light beam.

Expanding $\cos(\delta_0 \sin \omega t)$ in a Fourier series

$$I(t) = I_0 10^{-\bar{A}} \left[\cosh\left(\frac{LD \ln 10}{2}\right) + \sinh\left(\frac{LD \ln 10}{2}\right) \{J_0(\delta_0) + \sum_{n=1}^{\infty} 2J_{2n}(\delta_0) \cos(2n\omega t)\} \right] \quad (2.3)$$

$J_{2n}(\delta_0)$ are the Bessel functions of integer order. The photomultiplier current

$$i(t) \propto I(t) \quad (2.4)$$

With a phase sensitive lock-in amplifier the 2ω component

$$i_{AC} \propto 2 \sinh\left(\frac{LD \ln 10}{2}\right) J_2(\delta_0) \cos 2\omega t \quad (2.5)$$

is extracted. This signal is rectified and divided by i_{DC} so that

$$\frac{i_{AC}}{i_{DC}} = \frac{4J_2(\delta_0)}{\pi} \frac{\tanh\left(\frac{LD \ln 10}{2}\right)}{1 + J_0(\delta_0) \tanh\left(\frac{LD \ln 10}{2}\right)} \quad (2.6)$$

In practice i_{DC} is electronically kept at a constant level yielding

$$i_{AC} = K \tanh\left(\frac{LD \ln 10}{2}\right) \quad (2.7)$$

The instrumental constant K is determined experimentally (24,25) and the recorded linear dichroism

$$LD_r = \tanh\left(\frac{LD \ln 10}{2}\right) \quad (2.8)$$

For values of $|LD| < 0.1$ the recorded dichroism is directly proportional to the linear dichroism i.e. $LD_r = \frac{\ln 10}{2} LD$.

The principal set-up of an LD spectrometer is illustrated in Figure 2.1.

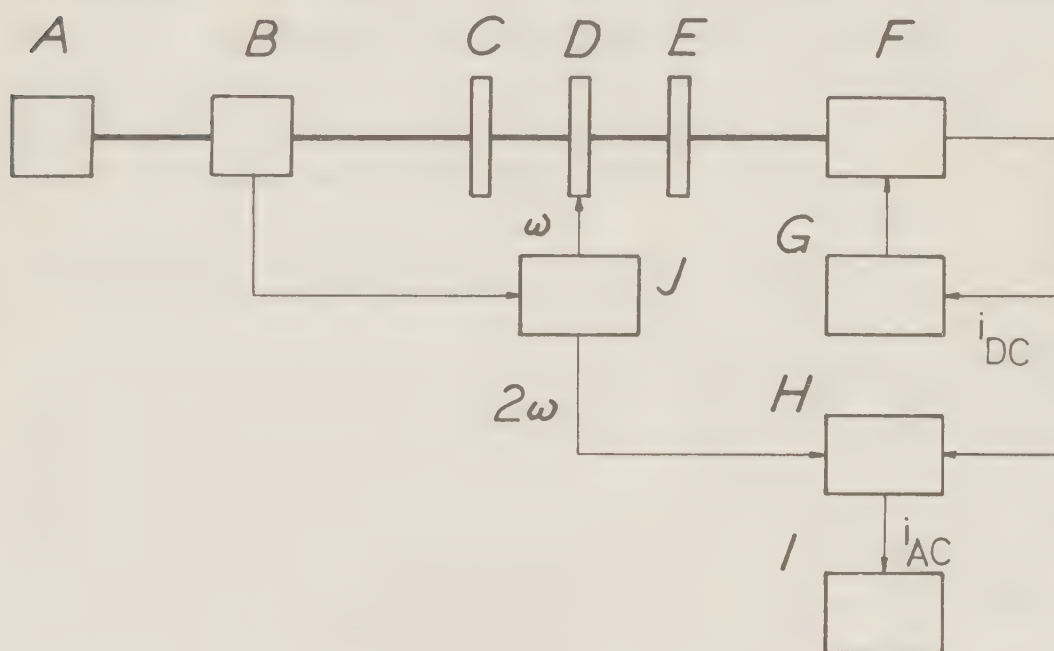


Figure 2.1. Schematic diagram of a LD spectrometer. A: light source, B: monochromator, C: linear polarizer, D: photoelastic modulator, E: sample, F: photomultiplier, G: high voltage servo, H: phase sensitive lock-in amplifier, I: recorder, J: modulator control.

The uniaxial anisotropic systems of interest here are membranes and lamellar liquid crystals containing chromophores. These systems are often macroscopically alignable in thin layers between quartz plates, so that the optical axis coincides with the normals of both lamellae and plates. Dichroism is therefore obtained only for an angle, $90^\circ - \omega$, of inclination between the impinging light beams and the normal, as illustrated in Figure 2.2. As shown in I

$$\frac{LD}{A_{\perp}} = \frac{A_{\omega} - A_{\perp}}{A_{\perp}} = \frac{3S}{1-S} \cos^2 \omega \quad (2.9)$$

A_{ω} and $A_{\perp}$ are the absorbances corresponding to the angles ω and 90° ($\perp$) between the polarization vector and the normal. The order parameter, $S = \frac{1}{2} \langle 3 \cos^2 \beta - 1 \rangle$, describes the average orientation of the transition dipole moment. β denotes the angle between the transition dipole and the normal of the bilayers i.e. the director.

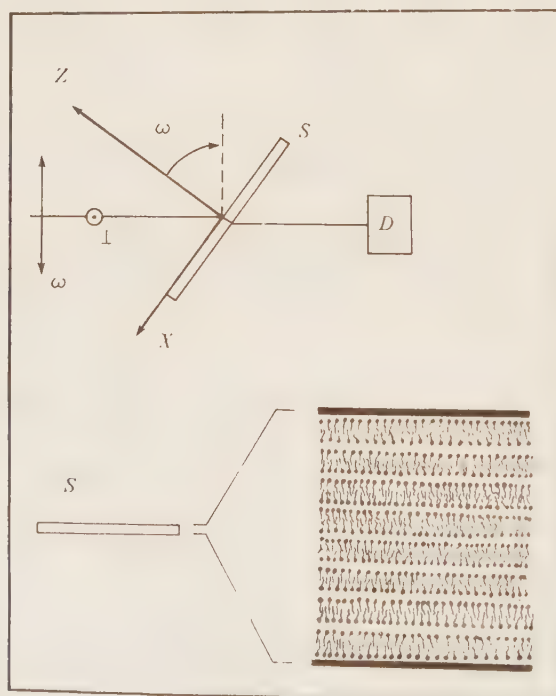


Figure 2.2. LD experiment: light with the directions of the polarization either ω or $\perp$ impinges on an aligned lamellar sample (S). The transmitted light is detected at D.

In practise eq. 2.9 cannot be used directly and the following corrections have to be done. The propagation direction of light impinging from air to sample medium will change due to refraction. A correction factor n^{-2} to $\cos^2 \omega$ is readily derived from Snell's law of refraction. n is the refractive index of the lamellae. In experiments where LD is measured directly the $A_{\perp}$ -spectra have here always been measured at normal incidence of light i.e. along the optical axis. The optical path lengths in the LD measurement is therefore a factor $(1-n^{-2}\cos^2 \omega)^{-\frac{1}{2}}$ longer than the path length in the $A_{\perp}$ measurement. Including both corrections eq. 2.9 becomes

$$\frac{LD}{A_{\perp}} = \frac{3S}{1-S} n^{-2} (1-n^{-2}\cos^2 \omega)^{-\frac{1}{2}} \cos^2 \omega \quad (2.10)$$

Taking the multiple reflections and the birefringence of an aligned sample into account, additional corrections of eq. 2.10 may be necessary. These complications are investigated and discussed in II. However, eq. 2.10 provides a good approximation for many systems such as membranes and model membranes.

2.2. Chromophore Orientation and Electronic Transitions

The linear dichroism depends on the orientational distribution of the chromophores, since the transition moments are polarized along fixed directions in the molecule. If these directions are known the average orientation of different molecular axes can be described. The order parameters convenient for this purpose are the Wigner rotation matrix elements, $\langle D_{nm}^{(2)*}(\alpha, \beta, \gamma) \rangle$. The brackets, $\langle \rangle$, denote a space average. For uniaxial systems (I)

$$\frac{LD}{A_{\perp}} = \frac{\sum_{n=-2}^2 P_n^M \langle D_{on}^{(2)*}(\beta\gamma) \rangle}{\text{Tr} \tilde{P} - \sum_{n=-2}^2 P_n^M \langle D_{on}^{(2)*}(\beta\gamma) \rangle} \quad (2.11)$$

P_n^M is an irreducible spherical tensor component of the molecular (M) absorptivity tensor $\tilde{P}$. The relations between P_n^M and the cartesian

components are given by eq. 2.5 in I.

Among the order parameters $\langle D_{00}^{(2)}(\beta) \rangle$ for example is the well known saupian order parameter, $S_{zz} = \frac{1}{2} \langle 3\cos^2\beta - 1 \rangle$, that describes the average orientation of the z-axis of the molecular frame. From eq. 2.11 it is obvious that knowledge of the P_n^M elements is a prerequisite for the evaluation of the order parameters.

For several chromophores of high symmetry assignments of transition moments have been made. Some examples are benzene (D_{6h} symmetry), tetraphenylporphyrin (D_{4h} symmetry) and anthracene (D_{2h} symmetry). However, most electron spectra of molecules of biological interest are still not resolved. Important examples are tryptophan, tyrosine, chlorophylls and further among the polyenes, ubiquinones and carotenoids. In the latter cases it appears to be a general opinion that the lowest energy transition is polarized parallel with the polyene chain. Although this seems reasonable it has not yet been verified quantitatively by experiments. The low symmetry of these chromophores makes the analysis more complicated (cf. Figure 2.3).

The isoalloxazine chromophores, acting in the prosthetic group of flavoproteins, also fall within this inconvenient category. Among the reduced, semiquinone and oxidized forms of isoalloxazine only the latter possesses a plane of symmetry. The absorption bands of the first three transitions of the oxidized isoalloxazine have been resolved by utilizing data obtained with X-ray, linear dichroism, electric linear dichroism and polarized fluorescence methods. These results are presented in III. In the following a general background to the need of several techniques is given.

Consider an anisotropic system of chromophores with a known uniaxial distribution. With $\frac{LD}{A_{\perp}}$ data the P_n^M elements can then be calculated by using eq. 2.11, since the order parameters are known. In planar chromophores the $\pi^* \leftarrow \pi$ transitions are generally polarized in this plane. To describe the polarization of such a transition with respect to an axis in the molecular plane, only one polar angle, θ , is needed. Eq. 2.11 therefore becomes a second order equation in $\sin\theta$ and $\cos\theta$.

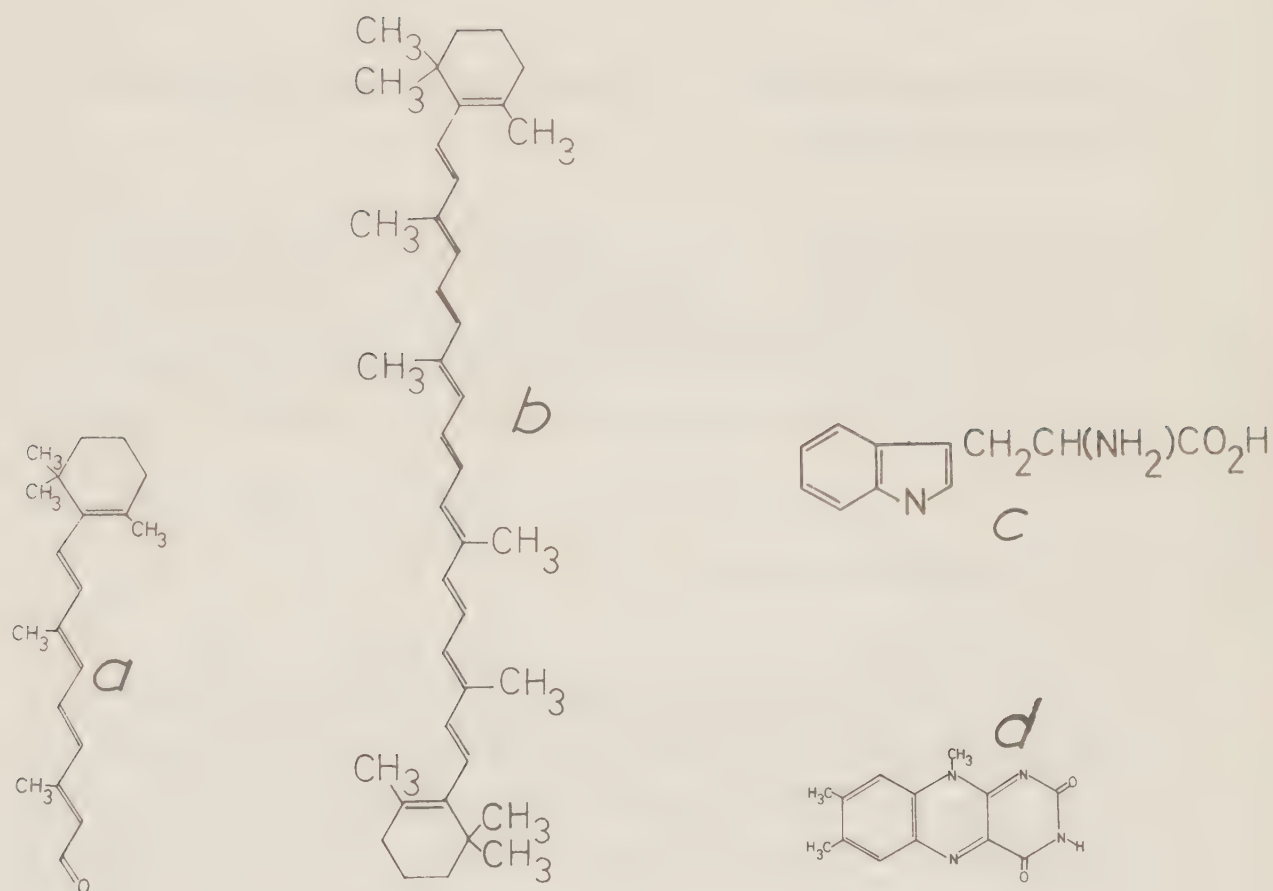


Figure 2.3. Structure formulas of a: all-trans retinal, b: trans-β-carotene, c: tryptophan, d: lumiflavin.

The problem is then to determine the correct one of the two possible angles obtained by solving this equation. This is feasible by studying the linear dichroism of two known and different chromophore distributions. One method is to incorporate the chromophore in a crystal lattice and determine its coordinates with X-ray techniques (26). Another method, which provides a permanent dipole moment in the molecules, is to orient them in an electric field. The electric linear dichroism method (ELD) exploits this idea (27). The polarized

fluorescence from isotropic solutions can sometimes give useful information in the discrimination among the angles. From the degree of polarization

$$p = \frac{3\cos^2\chi - 1}{\cos^2\chi + 3} \quad (2.12)$$

one can calculate the angle (χ) between the absorption and emission transition moments (28).

A combination of the data obtained from the various methods will in many cases reveal the correct transition moment directions in the chromophores. It should be noted that a knowledge of the polarizations are important not only in LD but even more crucial in the orientation studies where polarized fluorescence is used (cf. Section 4.2).

2.3. Orientation and Binding

The linear dichroism of chromophores distributed between the lipid and the water regions is investigated in V. The particular chromophores studied were the local anesthetics tetracaine and procaine. Tetracaine is amphiphilic with a solubility of about $10^{-2}M$ in water. The drug may therefore reside both in lipid bilayer and in the water regions. Since only the anisotropic fraction contributes to the linear dichroism information about the binding of the drug to the membrane might be obtained. Adopting a two site model of the distribution density function

$$f'(\beta) = pf(\beta) + (1 - p) \frac{1}{4\pi} \quad (2.13)$$

it follows (V) that

$$\frac{LD}{A_{\perp}} = \frac{3pS}{1 - pS} \cos^2\omega \quad (2.14)$$

where p stands for the anisotropic fraction of the chromophore.

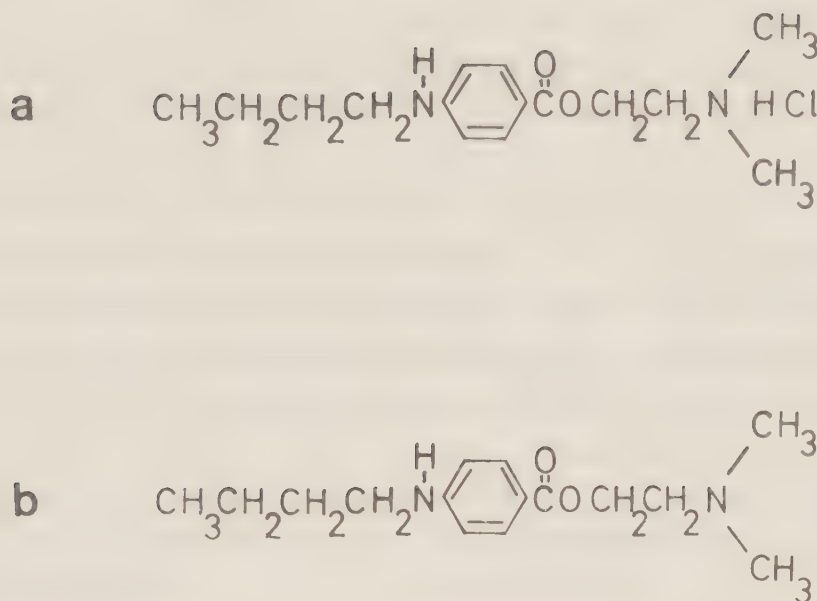


Figure 2.4. Structure formulae of a: tetracaine and b: tetracaine free base.

The crux is however to obtain p and S from the measured pS . One attempt to separate these factors could be to determine S of a much less water soluble but otherwise very similar chromophore (See Figure 2.4) or by utilizing a different independent method. The interpretation of the experimental results (V) by using this model leads to that $p \approx 1$ i.e. most of the tetracaine molecules are residing in the anisotropic region.

The product pS appears also in the NMR studies of water and ionbinding involving similar distributions. At present, however, there is no general approach to the problem of separating pS .

The organization of carotenoids present in both plant and animal membranes is far from clear. Among the carotenoids, β -carotene (Figure 2.3) has gained much attention. β -carotene is in animals the precursor of vitamin A (retinal) and is believed to act as a light protecting pigment in plants. Despite the lack of conclusive experimental evidence statement about that β -carotene orients with its long axis parallel to the acyl chains have been reported (29,30).

However, our linear dichroism study (IV) of β -carotene in different lipid bilayers contradicts such speculations. Instead the long axis of β -carotene is preferentially oriented perpendicular to the lipid acyl chains.

Table. The orderparameter, S , of β -carotene and retinal in different lamellar liquid crystalline samples at 22°C. The orderparameter describes the average orientation of the molecular long axis relative to the normal of the lamellae. Data from IV.

System	β -carotene Orderparameter S	all-trans-retinal Orderparameter S
1-Octylmonoglyceride -water; 70-30% w/w	- 0.46	0.28
Polyglycerol mono- laurate-water; 70-30% w/w	- 0.18	0.31
1-Oleoylmonoglyceride -water; 10-90% w/w	- 0.09	0.23
Dioleoylphosphatidyl- choline-water; 80-20% w/w	-0.22	0.23

3. PHASE STRUCTURES OF MAGNETICALLY ORIENTABLE LIQUID CRYSTALS

Lyotropic liquid crystalline phases that spontaneously orient in a magnetic field have been known for about 15 years (31,32). The phase structures of two such systems containing; sodium decylsulphate-sodium sulphate and potassium laurate-potassium chloride, both with heavy water are presented in VI and VII. The NMR measurements of the deuterium quadrupole splittings clearly show that the director in these systems is parallel to the applied magnetic field. For samples being macroscopically aligned between glass plates NMR reveals that the director is isotropically distributed in a plane parallel to the glass plates. Rodlike aggregates having their long axes parallel with the director, are compatible with this observation.

The rodlike structure is further supported by linear dichroism studies of solubilized all-trans-retinal which has similarities with an amphiphile as can be seen in Figure 2.3. Therefore one expects that in the aggregate retinal orients with its long axis preferentially parallel to the hydrocarbon chains, which is also observed (I, IV). In the lamellar phases of soaps an order parameter of about 0.4 is obtained. The order parameters of retinal found in the proposed rodlike aggregates are about -0.2. The ratios between the order parameters found in the magnetically orientable phases and in the lamellar phases are between -0.47 – -0.54 which is close to $-\frac{1}{2}$ expected entirely for geometrical reasons (VI).

A recently developed pulsed NMR method (5) for measuring the amphiphile translational diffusion within the aggregates has been utilized to estimate the lengths of the rods. The diffusion coefficient (D) along the rods was found to be about $10^{-11} \text{ m}^2/\text{s}$ for both systems. By using $\langle x^2 \rangle = 2Dt$ the lengths can be estimated to be longer than 1000 nm. Taken together all these experimental results strongly indicate that these systems consist of long rodlike aggregates.

A remarkable property of these systems is the finding that the macroscopical alignment of a sample may be kept over very long times. Although the aggregates orient in a magnetic field within an hour (at least for field strengths $\geq 2T$) no randomization can be observed even after about 100 hours. This together with the high transmission in the region $\sim 300 - 900$ nm make the systems very suitable as ordering matrices in optical spectroscopy.

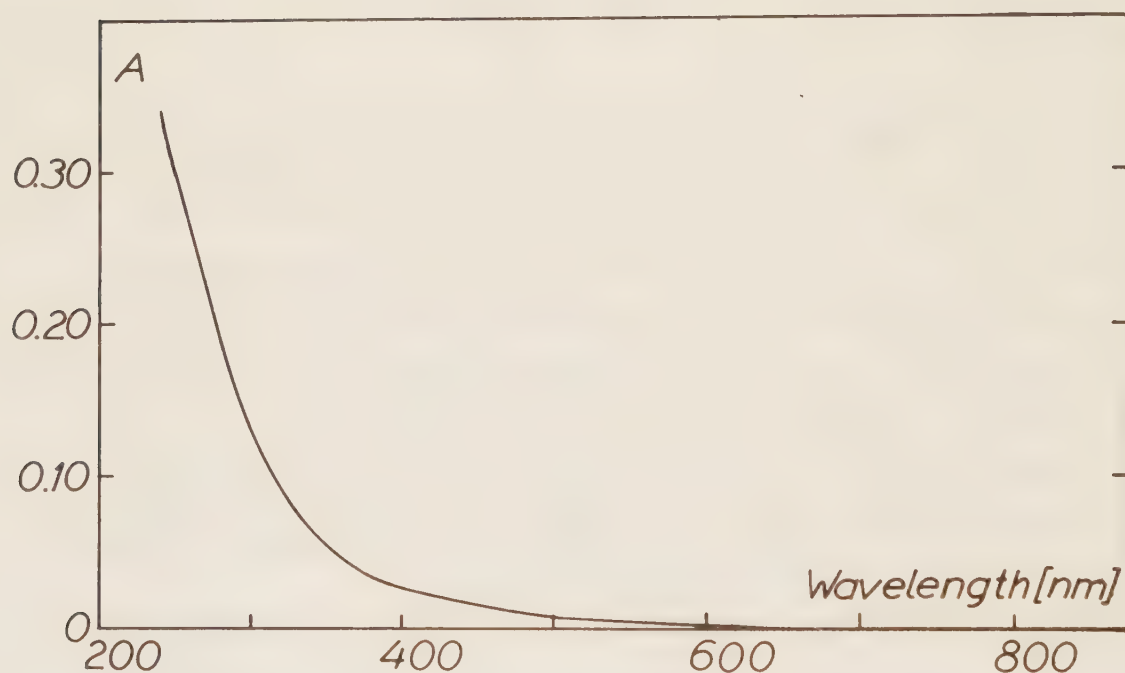


Figure 3.1. Absorption spectrum of the lyotropic liquid crystalline phase consisting of potassium laurate-potassium chloride-water. Pathlength 1 cm.

4. POLARIZED FLUORESCENCE IN MEMBRANE STUDIES

4.1. Fluorescence Detected Linear Dichroism

The study of chromophore orientation in membranes with linear dichroism requires macroscopical alignment of thin layers. The short optical path length might then cause sensitivity problems in investigations of e.g. membrane proteins, especially since these occur in very small amounts. Another problem is the overlap between the absorption spectra of different chromophores. This will generally make it impossible to interpret the linear dichroism.

The fluorescence emission from systems like micellar solutions or lamellar liquid crystalline phases, depend on both static and dynamic properties of the fluorophores. The static parameters are averaged Wigner rotation matrix elements and the dynamic ones involve the anisotropic motion and the fluorescence lifetime. The analytical expressions of the fluorescence intensity are then very complex and hardly no molecular information can in general be obtained from a macroscopically anisotropic system. For fluorophores that are immobile during the fluorescence lifetime one gets somewhat simpler expressions containing linear combinations of $\langle D_{on}^{(2)*}(\beta, \gamma) \rangle$ and $\langle D_{on}^{(4)*}(\beta, \gamma) \rangle$ (cf. 2.24-2.28 in I). These formulas are still very complicated but might sometimes be simplified so that $\langle D_{oo}^{(2)}(\beta) \rangle$ and $\langle D_{oo}^{(4)}(\beta) \rangle$ can be obtained (33). If, however, an intensity proportional to the total fluorescence emission, F , is measured the $\langle D_{on}^{(2)*}(\beta, \gamma) \rangle$ elements are conveniently extracted. No assumption about the rotational motion is neither necessary, which is shown in VIII. For an aligned membrane system, excited with the polarizations at the angles ω and $90^\circ(\perp)$ with respect to the director,

$$\frac{F_\omega}{F_\perp} = 1 + \frac{3 \sum_{n=-2}^2 P_n^M \langle D_{on}^{(2)*}(\beta, \gamma) \rangle \cos^2 \omega}{\text{Tr} \tilde{P} - \sum_{n=-2}^2 P_n^M \langle D_{on}^{(2)*}(\beta, \gamma) \rangle} \quad (4.1)$$

Except for the first term, 1, the right hand side of eq. 4.1 is identical to eq. 2.11 ($\cos^2\omega=1$). The fluorescence detected linear dichroism is therefore given by

$$\frac{FDLD}{A_{\perp}} = \frac{F_{\omega}}{F_{\perp}} - 1 \quad (4.2)$$

The derivations of these equations, the measuring of F and some experimental results are presented in VIII.

For experimental reasons fluorescence is much more sensitive than absorption. It is therefore very likely that FDLD will be far more sensitive than LD. Prerequisite conditions are, of course, highly fluorescent molecules such as tryptophan and flavins which are naturally occurring in many proteins. The fluorescence spectrum is most often shifted from the absorption spectrum towards lower energy. This might be utilized in orientation studies of a fluorophore whose absorption spectrum is overlapping with the spectra of other chromophores with negligible fluorescence. Hereby FDLD will be selective.

4.2 Time Resolved Fluorescence

Rather few studies of membranes utilizing time resolved fluorescence (TRF) have hitherto been published. The experimental work on liposomes containing solubilized fluorophores (34,35) strongly suggests that the method could be used to determine order parameters in macroscopically isotropic systems. In such systems the fluorescence anisotropy ($r(t_{\infty})$) of chromophores, which are solubilized in aggregates of amphiphiles (vesicles, micelles or cubic phases), is given by

$$r(t_{\infty}) = \frac{2}{5} \frac{\langle D_{00}^{(2)}(\Omega_{MD}) \rangle \sum_{n=-2}^2 P_n^M \langle D_{0n}^{(2)*}(\Omega_{MD}) \rangle}{\tilde{Tr}P} \quad (4.3)$$

This relation is derived in Appendix C of I. Ω_{MD} denotes the eulerian angles between the molecular frame (M) and the local director frame (D). The local director refers to the symmetry axis of the aggregates.

Note that the factor $\sum_n P_n^M \langle D_{on}^{(2)*}(\Omega_{MD}) \rangle$ also appears in the expression for LD in eq. 2.11. $\langle D_{oo}^{(2)}(\Omega_{MD}) \rangle$ is the order parameter of the emission transition moment. Since $r(t_\infty)$ contains products of different order parameters these cannot be obtained solely from this method. If however the directions of the absorption and the emission dipoles are known with respect to a molecular frame and these dipoles are parallel then

$$r(t_\infty) = \frac{2}{5} \langle D_{oo}^{(2)}(\Omega_{MD}) \rangle^2 \quad (4.4)$$

and $|\langle D_{oo}^{(2)}(\Omega_{MD}) \rangle|$ can be calculated. The knowledge of the transition moment directions in the molecule is hence a necessary demand in these studies.

TRF might also be a useful method in the determination of the symmetry of amphiphilic aggregates. This is feasible since the order parameter of an incorporated fluorophore is strongly depending on the aggregate symmetry. In a spherical micelle, for example the apparent order parameter should be twice as large as that for a rodlike micelle. The $r(t_\infty)$ value should then differ by a factor of four (I, 36).

The great advantage with the method is that the orientation of fluorophores solubilized in aggregates can be investigated in macroscopically isotropic systems. Efficiently fluorescent chromophores are then needed. Therefore the flavins (III) are expected to be very appropriate for TRF studies of for example proteins in lipid bilayers.

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I

Orientation and mobility of molecules in membranes studied by polarized light spectroscopy

LENNART B.-Å. JOHANSSON AND
 GÖRAN LINDBLOM

*Physical Chemistry 2, Chemical Centre, University of Lund,
 P.O. Box 740
 S-220 07 Lund 7, Sweden*

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I. INTRODUCTION

Biological membranes are composed of mainly lipids and proteins. The physical properties of the lipids, forming a bilayer structure, are of crucial importance for the living cell, since the plasma membrane is the guardian barrier towards the environment. Thus, the functioning cell needs a highly stable lipid bilayer, which depends on molecular packing and orientation properties of the various membrane components (Wieslander *et al.* 1980). The spatial arrangement of the membrane proteins incorporated in the lipid matrix plays an essential role for the different chemical processes occurring at or within the membrane. Information about molecular orientation and mobility is therefore necessary for unravelling the functional mechanisms of a biological membrane.

Many techniques based on polarized light spectroscopy have long been used for studying the orientation of chromophores in different systems, such as crystals, stretched polymer films, liquid crystals and membranes. Previously, Hofrichter & Eaton (1976) have reviewed polarized absorption investigations of the orientation of biological chromophores in various systems. The molecular orientation of a chromophore can be studied by the absorption or emission of polarized light of a macroscopically aligned sample. The orientation of the molecule is obtained, since the absorbance or emission measured depends on the polarization of the incident light and on the transition moments fixed in the molecule. Obviously, the direction of transition moments must be known and this information can often be obtained. A great advantage with polarized light spectroscopic methods is that they are very sensitive, compared with for example nuclear magnetic resonance (NMR), and usually tiny amounts of the biological sample can be studied. Furthermore, the orientation of the large membrane proteins can be determined. However, important information on the structure and dynamic behaviour of lipid systems has been obtained with NMR methods (Wennerström & Lindblom, 1977; Seelig, 1977, 1978) and further ionbinding and water orientation for model membrane systems have been reported (Lindblom Wennerström & Lindman, 1976*a*; Lindblom, Persson & Arvidson 1976*b*).

In this review we will concentrate on the use of polarized absorption and emission spectroscopy for studies of molecular orientation in membrane systems like bilayers, lyotropic liquid crystals and biogenic membranes. The anisotropic motion of molecules solubilized in the

membrane lipid matrix investigated by time resolved polarized fluorescence will also be discussed to some extent. It is not our intention, however, to give a complete review of the current literature but merely to follow our own interest and personal view on the subject.

Reading the literature in the field, where molecular orientation is to be obtained, we have the impression, first, that one is not always aware of the fact that a macroscopically aligned membrane system is a prerequisite for orientation studies with static polarized light spectroscopy; secondly, that the data obtained of *anisotropic* systems are not seldom inconsistently treated. The technical problem of macroscopically aligning samples will not be considered here (see, for example, Hofrichter & Eaton, 1976; Nordén, 1978) but a discussion of the basic principles seems appropriate and we will therefore present a formalism, which we have found to be very useful and convenient when considering the effects of anisotropy in both static and dynamic polarized light spectroscopy. This formalism is based on a matrix representation of the interaction between the linear polarized light and the chromophores. The very useful irreducible spherical tensor method has also been used for coordinate transformations in the anisotropic membrane systems (cf. Wennerström & Lindblom, 1977; Seelig, 1977). Further, we have had the intention of giving the most general formulae possible, so that these can be used for most conceivable cases.

Since the results obtained usually are interpreted in terms of order parameters a separate paragraph deals with the properties of these parameters. The part of application of polarized light spectroscopy will start with systems containing 'simple' molecules from a spectroscopical point of view, and then continue to sections discussing complex biological systems.

II. BASIC THEORY

(A) Polarized absorption on membranes

For most transitions in absorption and emission spectra (Dörr, 1966; Steinfeld, 1974) the interaction of molecules with electromagnetic radiation can be described by the effective Hamiltonian $\hat{H}' = \hat{\mathbf{p}} \cdot \mathbf{E}$, i.e. the interaction of the electric field, $\mathbf{E}$ with the dipole moment operator, $\hat{\mathbf{p}}$. In an experiment the absorbance, A is measured which is given by (Steinfeld, 1974):

$$A = k(\mathbf{p} \cdot \mathbf{E})^2, \quad (2.1)$$

where $\mathbf{p} = \langle \phi_i | \hat{\mathbf{p}} | \phi_i \rangle$ and k is a constant and it has been assumed that the population of the excited state is negligible compared with that of the ground state (i.e. a light source of moderate intensity is used). Equation (2.1) clearly shows that the absorbance will depend on the orientation of absorbing molecules.

The transition moment, $\mathbf{p}$, is regarded as a vector fixed in the molecule and the vector, $\mathbf{E}$ defines the linear polarization of light in a laboratory fixed coordinate system. Thus, in order to perform the scalar product in equation (2.1) the transition moment is transformed from the molecular frame (M) to the laboratory coordinate system (L). This is accomplished most conveniently by first mathematically forming a tensor $\tilde{\mathbf{P}}$ by a direct product between two $\mathbf{p}$ -vectors:

$$\tilde{\mathbf{P}} = \mathbf{p} \otimes \mathbf{p} = \begin{bmatrix} p_x^2 & p_x p_y & p_x p_z \\ p_x p_y & p_y^2 & p_y p_z \\ p_x p_z & p_y p_z & p_z^2 \end{bmatrix}. \quad (2.2)$$

The absorbance of a sample can then be written

$$A = k \mathbf{E} \tilde{\mathbf{P}} \mathbf{E}. \quad (2.3)$$

Representing the vector $\mathbf{E}$ by a (1×3) matrix, $\mathbf{E}$, and the tensor $\tilde{\mathbf{P}}$ by a (3×3) matrix, $\mathbb{P}$, equation (2.3) becomes

$$A = k \mathbf{E} L \mathbb{P} L^T \mathbf{E}, \quad (2.4)$$

where L stands for the transpose. The transformation of $\mathbb{P}^M$ to $\mathbb{P}^L$ can, of course, be performed by using a Cartesian transformation tensor, although somewhat cumbersome, as described in the review by Hofrichter & Eaton (1976). Here we will instead use irreducible spherical tensors, since the different tensor components have simple transformation properties under rotation of the coordinate system. Such a rotation is defined by a set of eulerian angles $\alpha, \beta, \gamma \equiv \Omega$. The nine Cartesian components of $\mathbb{P}^L$ are related to the corresponding five irreducible components $P_{\pm 2}^L, P_{\pm 1}^L$ and P_0^L (see Appendix A) according to:

$$P_{XX}^L = \frac{1}{3} \text{Tr } \tilde{\mathbf{P}} + \frac{1}{\sqrt{6}} (P_2^L + P_{-2}^L) - \frac{1}{3} P_0^L,$$

$$P_{YY}^L = \frac{1}{3} \text{Tr } \tilde{\mathbf{P}} - \frac{1}{\sqrt{6}} (P_2^L + P_{-2}^L) - \frac{1}{3} P_0^L,$$

$$P_{ZZ}^L = \frac{1}{3} \text{Tr } \tilde{\mathbf{P}} + \frac{2}{3} P_0^L,$$

$$P_{XZ}^L = -\frac{i}{\sqrt{6}} (P_{+2}^L - P_{-2}^L),$$

$$P_{YZ}^L = \frac{i}{\sqrt{6}} (P_{+1}^L + P_{-1}^L),$$

$$P_{xz}^L = -\frac{1}{\sqrt{6}}(P_{+1}^L - P_{-1}^L),$$

$$\text{Tr } \tilde{P} = P_{xx}^L + P_{yy}^L + P_{zz}^L = P_{xx}^M + P_{yy}^M + P_{zz}^M. \quad (2.5)$$

The transformation of the spherical tensor elements P_n^M to an element P_n^L is given by

$$P_n^L = \sum_{m=-2}^2 D_{nm}^{(2)*}(\Omega) P_m^M, \quad (2.6)$$

where $D_{nm}^{(2)*}$ is the complex conjugate of the Wigner rotation matrix element $D_{nm}^{(2)}$ (Brink & Satchler, 1968). A list of these second rank elements, of interest here, is given in the Appendix A.

Let us now consider the absorption of polarized light of an anisotropic sample like a lamellar liquid crystal, which has been macroscopically aligned. There is cylindrical symmetry about the normal to the lamellae an axis which often is called the director. This is taken to be the Z-axis of the laboratory coordinate system. The cylindrically symmetric distribution of molecules around the laboratory Z-axis then leads to an averaging of the $\mathbb{P}^L$ matrix elements, performed by taking the average value of the rotation matrix elements as

$$\langle D_{nm}^{(2)*} \rangle = \int_{\Omega} D_{nm}^{(2)*}(\Omega) f(\Omega) d\Omega, \quad (2.7)$$

where $d\Omega = d\alpha \sin \beta d\beta d\gamma$ and $f(\Omega)$ is proportional to the probability that the molecule has the orientation Ω . Now, since the system has a k -fold (k larger than 2) symmetry axis Z we have that

$$f(\alpha\beta\gamma) = f\left(\alpha + \frac{2h\pi}{k}, \beta, \gamma\right) = f(\beta\gamma) \quad (h = 1, 2, \dots, k). \quad (2.8)$$

Taking the average over α then gives, since

$$D_{nm}^{(2)*}(\Omega) = e^{ima} d_{mn}^{(2)}(\beta) e^{im\gamma} \quad (2.9)$$

the following integral $\int_0^{2\pi} e^{ima} d\alpha$.

which will vanish unless m is zero.

The mean value of the Cartesian $\mathbb{P}^L$ elements in (2.5) then gives with equation (2.6) and (2.10)

$$\langle \mathbb{P}^L \rangle = \begin{pmatrix} \frac{1}{3} \text{Tr } \tilde{P} - \frac{1}{3} \sum_n \langle D_{0n}^{(2)*}(\beta\gamma) \rangle P_n^M & 0 & 0 \\ 0 & \frac{1}{3} \text{Tr } \tilde{P} - \frac{1}{3} \sum_n \langle D_{0n}^{(2)*}(\beta\gamma) \rangle P_n^M & 0 \\ 0 & 0 & \frac{1}{3} \text{Tr } \tilde{P} + \frac{2}{3} \sum_n \langle D_{0n}^{(2)*}(\beta\gamma) \rangle P_n^M \end{pmatrix} \quad (2.11)$$

This matrix then describes the anisotropic absorption of any system with uniaxial symmetry. Note that this *effective* absorptivity tensor $\langle \mathbb{P}^L \rangle$ has some special properties. As can be inferred from equation (2.11) it is axial symmetric, having diagonal elements only and can be written

$$\langle \mathbb{P}^L \rangle = \begin{pmatrix} P_{\perp} & 0 & 0 \\ 0 & P_{\perp} & 0 \\ 0 & 0 & P_{\parallel} \end{pmatrix}. \quad (2.12)$$

Thus a molecule of any symmetry dissolved in a uniaxial system, will effectively act as a cylindrically symmetric pseudomolecule having transition moments only parallel ($\parallel$) and perpendicular ($\perp$) to the symmetry axis (the director). This property also leads to that the isotropic absorbance of such an anisotropic system can be measured in a single absorption experiment. The isotropic absorbance is determined by the trace of the ensemble averaged absorptivity tensor; i.e. $\text{Tr } \langle \mathbb{P}^L \rangle = \text{Tr } \tilde{P} = |\mathbf{p}|^2$. This also follows from the fact that the trace is independent of the coordinate system. Thus if the polarization of the linear polarized light are chosen so that the $\text{Tr } \tilde{P}$ is obtained, then the isotropic absorbance can be determined, and one such possible choice of polarization is with

$$\mathbb{E}^L = \begin{pmatrix} 2 & 1 \\ \sqrt{3} & \sqrt{3} \end{pmatrix}.$$

In this review we will consider a special kind of uniaxial systems namely; membranes and lyotropic liquid crystals. For such a system, usually macroscopically aligned, e.g. between quartz plates, the director coincides with the normal of the plates or the lipid bilayer. Thus to be able to study molecular orientation by polarized absorption then the direction of the incident light cannot be along the normal of the plates (Fig. 1).

For an angle $(\pi/2) - \omega$ of incidence with respect to the normal of the plates (see Fig. 1) and with the electric field vector either perpendicular ($\perp$) or at an angle ω with respect to the director, the corresponding absorbances become

$$A_{\perp} = k \langle \mathbf{o} \mid \mathbf{o} \rangle \langle \mathbb{P}^L \rangle \langle \mathbf{o} \mid \mathbf{o} \rangle^t = k \left(\frac{1}{3} \text{Tr } \tilde{P} - \frac{1}{3} \sum_n \langle D_{0n}^{(2)*}(\beta\gamma) \rangle P_n^M \right) \quad (2.13)$$

$$\begin{aligned} A_{\omega} &= k \langle (-\sin \omega \mid \mathbf{o} \cos \omega) \langle \mathbb{P}^L \rangle (-\sin \omega \mid \mathbf{o} \cos \omega) \rangle^t \\ &= k \left(\sum_n \langle D_{0n}^{(2)*}(\beta\gamma) \rangle P_n^M \cos^2 \omega + \frac{1}{3} \text{Tr } \tilde{P} - \frac{1}{3} \sum_n \langle D_{0n}^{(2)*}(\beta\gamma) \rangle P_n^M \right). \end{aligned} \quad (2.14)$$

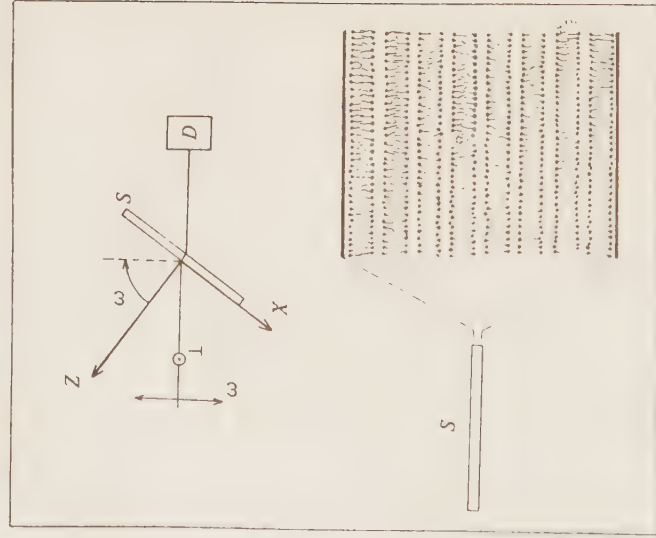


Fig. 1. Polarized absorption measurements: light with the polarization directions ω or $\perp$ impinges on an aligned lamellar sample (S). The transmitted light is detected at D .

Experimentally $A_{\perp}$ and A_{ω} can be determined from two separate experiments by appropriate rotations of a linear polarizer. When this method is used it is convenient to define a dichroic ratio D as

$$D = \frac{A_{\omega}}{A_{\perp}} = 1 + \frac{3 \sum_n \langle D_{0n}^{(2)*}(\beta\gamma) \rangle P_n^M \cos^2 \omega}{\text{Tr} \bar{P} - \sum_n \langle D_{0n}^{(2)*}(\beta\gamma) \rangle P_n^M} \quad (2.15)$$

A more sensitive technique is offered by the use of photoelastic modulators in combination with a lock-in phase detector (Badoz *et al.* 1977; Cheng, 1977; Hipps & Crosby, 1979) the linear dichroism $LD = A_{\omega} - A_{\perp}$ can be measured directly, and

$$\frac{LD}{A_{\perp}} = \frac{3 \sum_n \langle D_{0n}^{(2)*}(\beta\gamma) \rangle P_n^M \cos^2 \omega}{\text{Tr} \bar{P} - \sum_n \langle D_{0n}^{(2)*}(\beta\gamma) \rangle P_n^M} \quad (2.16)$$

If only *one* transition moment is considered with the molecular z -axis

parallel to it, n is equal to zero in the equations (2.15) and (2.16). Thus

$$D = 1 + \frac{3 \langle D_{00}^{(2)*}(\beta) \rangle \cos^2 \omega}{1 - \langle D_{00}^{(2)*}(\beta) \rangle} = 1 + \frac{3S \cos^2 \omega}{1 - S} \quad (2.17)$$

and

$$\frac{LD}{A_{\perp}} = \frac{3 \langle D_{00}^{(2)*}(\beta) \rangle \cos^2 \omega}{1 - \langle D_{00}^{(2)*}(\beta) \rangle} = \frac{3S}{1 - S} \cos^2 \omega, \quad (2.18)$$

where S is the more familiar order parameter (see Section III) introduced by Saupe (1964).

(B) Polarized emission of immobile molecules in membranes

In a fluorescence experiment the following steps occur. Light impinging on a molecule is absorbed with a probability proportional to $|\mathbf{p} \cdot \mathbf{E}|^2$. The absorption process is then followed by intramolecular processes that often result in fluorescence characterized by an emission transition dipole $\mathbf{q}$. The probability of emission of light is then proportional to $|\mathbf{q} \cdot \mathbf{E}'|^2$, where $\mathbf{E}'$ is the electric field vector of the emitted wave. The intensity of the fluorescence can be expressed in the form

$$F = k'(\mathbf{p} \cdot \mathbf{E})^2(\mathbf{q} \cdot \mathbf{E}')^2, \quad (2.19)$$

where k' is a constant.

Generally, the transition moment vectors $\mathbf{p}$ and $\mathbf{q}$ are not parallel, but form an angle, δ , with each other.

Let us now consider the fluorescence from an ensemble of molecules in a uniaxial system. This subject has been treated previously by several authors (Cehelnik *et al.* 1975; Cehelnik, Mielenz & Cundall, 1976; Chapoy & DuPré, 1978) although somewhat differently from our approach. In the following derivation two main assumptions will be made, namely that the excited molecules undergo negligible rotational Brownian motion during their lifetime (the rotational motion will be considered in Section V) and further that no photochemical processes (e.g. eximer, exciplex formation) occur. Usually these assumptions can be tested experimentally.

As in the previous section the macroscopically aligned membrane system is in a laboratory coordinate system (L), XYZ (Fig. 2) where the director coincide with the Z -axis. Plane polarized light propagates in the XZ -plane forming an angle $(\pi/2) - \omega$ with the Z -axis. The emitted light is then detected at an angle, θ , with respect to the Z -axis, after

passing a linear polarizer having the transmission axis characterized by $\mathbb{T}^L$. In analogy with the previous treatment of the absorbance (A) of an anisotropic system we get for the fluorescence

$$F = k'' A \mathbb{T}^L Q^L \mathbb{T}^L, \quad (2.20)$$

where Q stands for the emission transition moment matrix formed from the direct product $\hat{Q} = \mathbf{q} \otimes \mathbf{q}$. The elements of Q^L can thus be obtained by replacing $P_{ij}^L \rightarrow Q_{ij}^L$ and $P_n^M \rightarrow Q_n^M$ in equations (2.5) and (2.6). There are mainly two ways of exciting an anisotropic system namely to choose the polarization of the exciting electric field vector to be either perpendicular or making an angle ω with the director. Thus we need the following ensemble mean values

$$\langle F_\omega \rangle = \langle A_\omega Q^L \rangle, \quad \langle F_\perp \rangle = \langle A_\perp Q^L \rangle, \quad (2.21)$$

where

$$\langle F_{ij\omega} \rangle = \int_0^\pi \int_0^{2\pi} (P_{XX}^L \sin^2 \omega + P_{ZZ}^L \cos^2 \omega - P_{XX}^L \sin 2\omega) \times Q_{ij}^L f(\beta\gamma) \sin \beta d\beta d\alpha d\gamma, \quad (2.22)$$

$$\langle F_{ij\perp} \rangle = \int_0^\pi \int_0^{2\pi} P_{YZ}^L Q_{ij}^L f(\beta\gamma) \sin \beta d\beta d\alpha d\gamma. \quad (2.23)$$

Note that the mean value must be taken over the product AQ^L , i.e. it is usually not possible to first take the average of A and use the equations (2.13) and (2.14). The space averages of (2.22) and (2.23) are given in Appendix B for the case with the molecular z -axis parallel to the emission transition dipole moment.

In a polarized fluorescence experiment usually the degree of polarization, p is measured. This quantity is defined by:

$$p = \frac{F_\theta - F_\perp}{F_\theta + F_\perp}, \quad (2.24)$$

where F_θ and $F_\perp$ are the fluorescent intensities obtained with the transmission axis of the polarizer at an angle $(\pi/2) - \theta$ or perpendicular to the director, respectively. See Fig. 2. When the polarization of the exciting light is perpendicular to the director equation (2.20)–(2.24) yield

$$p_\perp = \frac{2 \text{Tr} \tilde{P} \langle D_{00}^{(2)} \rangle \cos^2 \theta + \sum_m (a_m \sin^2 \theta + c_m \cos^2 \theta - b_m) P_m^M}{\frac{2}{3} \text{Tr} \tilde{P} \{ 2 + \langle D_{00}^{(2)} \rangle (3 \cos^2 \theta - 2) \} + \sum_m (b_m + a_m \sin^2 \theta + c_m \cos^2 \theta) P_m^M}, \quad (2.25)$$

a_m , b_m and c_m are constants which are linear combinations of $\langle D_{0m}^{(2)*} \rangle$ and $\langle D_{0m}^{(2)} \rangle$. These constants are tabulated in Appendix B.

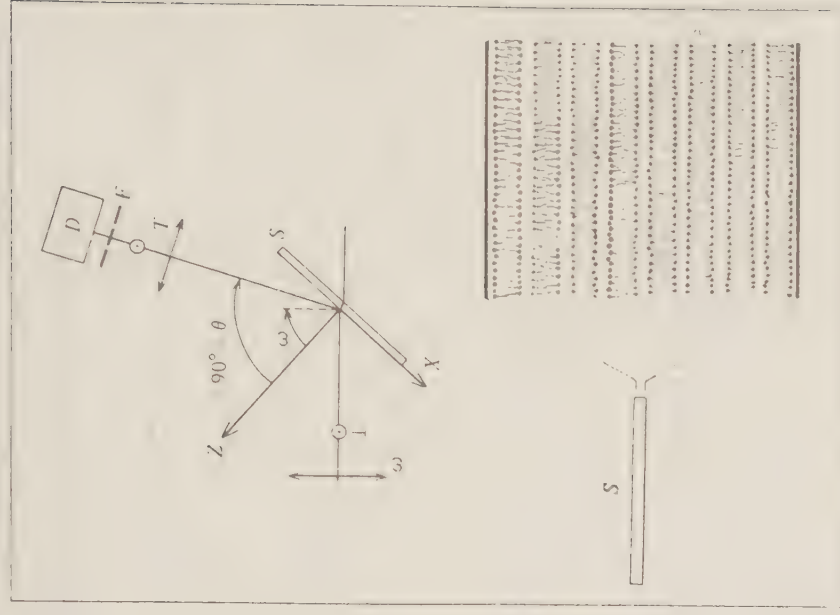


Fig. 2. Polarized emission measurement: light with the polarization directions either ω or $\perp$ impinges on an orientated lamellar sample (S). The emitted light is detected (D) after first passing a linear polarizer (the analyser denoted by T) and then a cut-off filter F .

The expression for the degree of polarization p_ω , i.e. with the exciting light vector at an angle ω with the director is $p_\omega = N/D$,

$$N = 2 \text{Tr} \tilde{P} \langle D_{00}^{(2)} \rangle \cos^2 \theta + \sum_m \{ A_m \sin^2 \omega \sin^2 \theta + (B_m \sin^2 \omega + C_m \cos^2 \omega) \times \cos^2 \theta + d_m \sin 2\omega \sin 2\theta \} P_m^M, \quad (2.26)$$

$$D = \frac{2}{3} \text{Tr} \tilde{P} \{ 2 + \langle D_{00}^{(2)} \rangle (3 \cos^2 \theta - 2) \} + \sum_m \{ (D_m \sin^2 \omega + E_m \cos^2 \omega) \sin^2 \theta + (F_m \sin^2 \omega + G_m \cos^2 \omega) \cos^2 \theta + d_m \sin 2\omega \sin 2\theta \} P_m^M. \quad (2.27)$$

The coefficients $A_m, B_m, \dots, G_m$ and d_m are given in Appendix B. Notice

that p contains ten order parameters; five from $\langle D_{0n}^{(2)*} \rangle$ and five from $\langle D_{0n}^{(4)*} \rangle$. Thus ten independent observations will be needed to determine all the then parameters. Two such independent observations are $p_{\perp}$ and p_{ω} . If these quantities could be determined for five known different dipole transition moment directions, then in principal all ten order parameters can be determined. So far molecular symmetry has not been taken into account. If this is done the number of order parameters needed for a description of the molecular orientation will be reduced most considerably. For molecules containing a plane of symmetry there are six independent unknown parameters since, $\langle D_{0n}^{(k)*} \rangle = (-1)^n \langle D_{0n}^{(k)*} \rangle$ (k even). When the molecular symmetry is higher than C_{2v} the number is reduced to four since $\langle D_{0n}^{(k)*} \rangle = (-1)^n \langle D_{0-n}^{(k)*} \rangle$ (k even). Molecules having a $(k+1)$ -fold or higher axis of rotation can be regarded as cylindrically symmetric, i.e. $\langle D_{0n}^{(k)*} \rangle$ vanishes unless n is equal to zero, and only $\langle D_{00}^{(2)} \rangle$ and $\langle D_{00}^{(4)} \rangle$ will remain. The degree of polarization will thus for the case of cylindrically symmetric molecules excited and studied with the polarizer settings perpendicular and parallel (i.e. $\theta = \omega = 0$) to the director take the most simple forms:

$$p_{\perp} = \frac{\langle D_{00}^{(2)}(\beta) \rangle - \langle \frac{2}{3} + \frac{3}{5} D_{00}^{(2)}(\beta) \rangle D_{00}^{(2)}(\delta)}{\frac{1}{3} \langle 2 + D_{00}^{(2)}(\beta) \rangle + \langle \frac{2}{15} - \frac{3}{2} D_{00}^{(2)}(\beta) - \frac{3}{5} D_{00}^{(4)}(\beta) \rangle D_{00}^{(2)}(\delta)}, \quad (2.27)$$

$$p_{\omega=0} = \frac{\langle D_{00}^{(2)}(\beta) \rangle - \langle \frac{2}{3} + \frac{4}{7} D_{00}^{(2)}(\beta) + \frac{3}{35} D_{00}^{(4)}(\beta) \rangle D_{00}^{(2)}(\delta)}{\frac{1}{3} \langle 2 + D_{00}^{(2)}(\beta) \rangle + \langle \frac{2}{15} + \frac{3}{2} D_{00}^{(2)}(\beta) + \frac{12}{35} D_{00}^{(4)}(\beta) \rangle D_{00}^{(2)}(\delta)}. \quad (2.28)$$

A similar equation of the fluorescence anisotropy expressed in terms of $\langle \cos^4 \beta \rangle$ and $\langle \cos^2 \beta \rangle$ was recently given by Chapoy & DuPré (1978). As can be inferred from equations (2.27) and (2.28) both $\langle D_{00}^{(2)}(\beta) \rangle$ and $\langle D_{00}^{(4)}(\beta) \rangle$ elements can be determined from the two independent experiments. However if the $\langle D_{00}^{(2)}(\beta) \rangle$ element is determined from polarized absorption only one fluorescence experiment, $p_{\perp}$ or p_{ω} , is necessary. Another possibility to determine the second rank elements is to measure the *total* emitted intensity from the sample or any intensity proportional to that (Johansson & Lindblom, 1980a). If this intensity is denoted by, F_t , the $\langle D_{00}^{(2)}(\beta) \rangle$ element can be obtained from

$$D = \frac{F_{\omega,t}}{F_{\perp,t}} = \frac{\text{Tr} \langle F_{\omega} \rangle}{\text{Tr} \langle F_{\perp} \rangle} = 1 + \frac{3 \sum_n \langle D_{0n}^{(2)*}(\beta\gamma) \rangle P_n^M \cos^2 \omega}{\text{Tr} \tilde{P} - \sum_n \langle D_{0n}^{(2)*}(\beta\gamma) \rangle P_n^M} \quad (2.29)$$

$$\begin{aligned} \frac{LD}{A_{\perp}} &= \frac{F_{\omega,t} - F_{\perp,t}}{F_{\perp,t}} = \frac{\text{Tr} \langle F_{\omega} \rangle - \text{Tr} \langle F_{\perp} \rangle}{\text{Tr} \langle F_{\perp} \rangle} \\ &= \frac{3 \sum_n \langle D_{0n}^{(2)*}(\beta\gamma) \rangle P_n^M \cos^2 \omega}{\text{Tr} \tilde{P} - \sum_n \langle D_{0n}^{(2)*}(\beta\gamma) \rangle P_n^M}. \end{aligned} \quad (2.30)$$

Yet another method to obtain the second rank elements based on time resolved fluorescence is outlined in V. A.

III. ORDER PARAMETERS AND MOLECULAR ALIGNMENT

The ensemble averaged Wagner rotation matrix elements $\langle D_{0n}^{(2)*}(\beta\gamma) \rangle$ and $\langle D_{0n}^{(4)*}(\beta\gamma) \rangle$ describe the molecular orientation and the averages of the second order D -elements are also closely related to the order parameters (cf. Appendix A). Now, all these averaged rotation matrix elements of second and fourth rank are in turn determined by the orientational distribution function and we will therefore discuss the properties of this function. A knowledge of the orientational distribution function is of great importance in the various theories describing the properties of liquid crystalline systems (Marčelja, 1974; Jähnig, 1979a).

The mean orientation of a molecule in a membrane system can be described by a variety of functions. It is convenient to expand the distribution density function in terms of a complete basis set of functions, as for example the Wigner matrices:

$$f(\Omega) = \sum_{k,m,n} \frac{2k+1}{8\pi^2} C_{mn}^{(k)} D_{mn}^{(k)}(\Omega), \quad (3.1)$$

where $C_{mn}^{(k)}$ are independent of the Eulerian angles. These coefficients are known as the motional constants. By using equation (2.7) it can be shown that the constants, $C_{mn}^{(k)}$, are directly proportional to the averaged rotation matrices with the same rank:

$$\langle D_{0n}^{(k)*}(\beta\gamma) \rangle = \int_{\Omega} D_{0n}^{(k)*}(\beta\gamma) \sum_n \frac{2k+1}{8\pi^2} C_{0n}^{(k)} D_{0n}^{(k)}(\beta\gamma) d\Omega. \quad (3.2)$$

Making use of the orthonormality relation of the Wigner matrices one obtains

$$C_{0n}^{(k)} = \langle D_{0n}^{(k)*}(\beta\gamma) \rangle. \quad (3.3)$$

The distribution function can then be written

$$f(\beta\gamma) = \frac{1}{4\pi^2} \left\{ \frac{1}{2} + \sum_{k \neq 0} \left(\frac{2k+1}{2} \right) \langle D_{0n}^{(k)*}(\beta\gamma) \rangle D_{0n}^{(k)}(\beta\gamma) \right\}, \quad (3.4)$$

where we have used that $D_{00}^{(0)} = 1$. The membrane symmetry, $D_{\infty h}$ (in the absence of chiral molecules), leads to that

$$\langle D_{0n}^{(k)*}(\beta, \gamma) \rangle = (-1)^k \langle D_{0n}^{(k)*}(\beta, \gamma) \rangle,$$

i.e. $\langle D_{0n}^{(k)*}(\beta, \gamma) \rangle = 0$ for all odd k . A polarized absorption experiment can only provide the coefficients of second order in the expansion of $f(\beta\gamma)$ in (3.4). Generally the distribution function as given by (3.1) does not converge rapidly. Thus truncation of the series after only the second term (k , even) leads to a rather poor representation of the real distribution density function. An improvement of the experimental description of $f(\beta\gamma)$ can, however, be obtained from polarized fluorescence or Raman measurements, where also the elements of fourth order can be determined. Sometimes these terms can also be obtained from an appropriate analysis of electron spin resonance data (Luckhurst *et al.*, 1979). The orientational distribution function that can be obtained from experiment on a chromophore of arbitrary symmetry with the molecular x -axis parallel to the transition dipole moment takes the form

$$f(\beta) = \frac{1}{2\pi} \left(\frac{1}{2} + \frac{5}{2} \langle D_{00}^{(2)}(\beta) \rangle D_{00}^{(2)}(\beta) + \frac{9}{2} \langle D_{00}^{(4)}(\beta) \rangle D_{00}^{(4)}(\beta) \right). \quad (3.5)$$

The range of definition of the second rank rotation matrix elements is $-\frac{1}{2} \leq \langle D_{00}^{(2)}(\beta) \rangle \leq 1$. When $\langle D_{00}^{(2)}(\beta) \rangle = 1$, the molecular z -axis is parallel to the director and when this element is $-\frac{1}{2}$ it is perpendicular to it. For these extreme values, of course, all molecules in the liquid crystalline phase have exactly the same alignment, i.e. $\beta = 0(\pi)$ or $\pi/2$, respectively. The distribution function is then a Dirac delta function.

Figure 3 illustrates the behaviour of the function $f(\beta)$, terminated after the second-order term, $f^{(2)}(\beta)$. Note that $f^{(2)}(\beta)$ has its maxima at $\beta = \frac{1}{2}\pi$ for $\langle D_{00}^{(2)}(\beta) \rangle < 0$ and at $\beta = 0$ or π for $\langle D_{00}^{(2)}(\beta) \rangle > 0$. This appearance of $f^{(2)}(\beta)$ is often referred to in common language as that the molecule tends to be orientated parallel (positive-order parameter) or perpendicular (negative-order parameter) relative to the director. Since we cannot measure higher terms than of rank four we ask what kind of improvement will a truncation after the $D^{(4)}$ terms lead to? Obviously, the sign of the terms $\langle D_{00}^{(2)}(\beta) \rangle$ and $\langle D_{00}^{(4)}(\beta) \rangle$ will have an influence on the magnitude of the distribution density function in the interval. It can thus be shown that in all cases but one gives the sign of the order parameter, $\langle D_{00}^{(2)}(\beta) \rangle$, a correct qualitative description of the molecular alignment in a uniaxial system. In Fig. 4 orientational functions $f(\beta)$ are plotted for various values and signs of the order parameters of second and fourth orders.

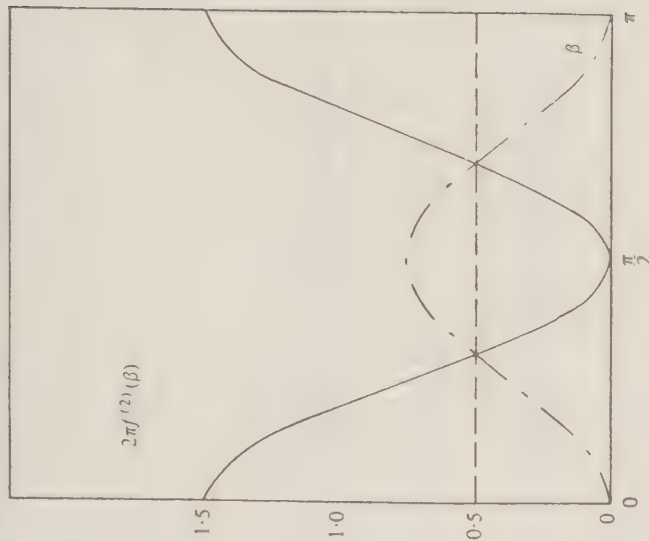


Fig. 3. The distribution density function $f^{(2)}(\beta)$, calculated by using the first two non-vanishing elements of the power-expansion

$$f(\beta) = \frac{1}{2\pi} \sum_0^{2k+1} \frac{\langle D_{00}^{(k)}(\beta) \rangle D_{00}^{(k)}(\beta)}{2}.$$

$$\langle D_{00}^{(0)}(\beta) \rangle = 1 \text{ and } \langle D_{00}^{(2)}(\beta) \rangle = 0.4 \text{ (—), } \langle D_{00}^{(2)}(\beta) \rangle = 0 \text{ (---), } \langle D_{00}^{(2)}(\beta) \rangle = -0.2 \text{ (-.-.-).}$$

For the case $\langle D_{00}^{(2)}(\beta) \rangle > 0$ and $\langle D_{00}^{(4)}(\beta) \rangle < 0$ it is found that as long as $\langle D_{00}^{(2)}(\beta) \rangle > -6 \langle D_{00}^{(4)}(\beta) \rangle$ the maximum of $f(\beta)$ are at $\beta = 0$ and π but at

$$\beta_{\max} = \arccos \pm \sqrt{\frac{1}{7} \left(3 - \frac{2 \langle D_{00}^{(2)}(\beta) \rangle}{3 \langle D_{00}^{(4)}(\beta) \rangle} \right)} \quad \text{otherwise (see Fig. 4a).}$$

When both $\langle D_{00}^{(2)}(\beta) \rangle$ and $\langle D_{00}^{(4)}(\beta) \rangle$ are negative the $f(\beta)$ maximum is always obtained at this $\beta_{\max}$ (Fig. 4b). However, when $\langle D_{00}^{(2)}(\beta) \rangle < 0$ and $\langle D_{00}^{(4)}(\beta) \rangle > 0$ a different situation may occur. As long as

$$\langle D_{00}^{(2)}(\beta) \rangle < -0.75 \langle D_{00}^{(4)}(\beta) \rangle$$

$f(\beta)$ will have its maximum at the 'expected' $\beta = \frac{1}{2}\pi$ but if

$$\langle D_{00}^{(2)}(\beta) \rangle > -0.75 \langle D_{00}^{(4)}(\beta) \rangle,$$

the maxima are at $\beta = 0$ or π . Thus in this latter case our qualitative interpretation of a negative-order parameter, $\langle D_{00}^{(2)}(\beta) \rangle$ will be wrong. An example of this is shown in Fig. 4c, where it can be seen that the probability of finding a molecule orientated parallel to the director is

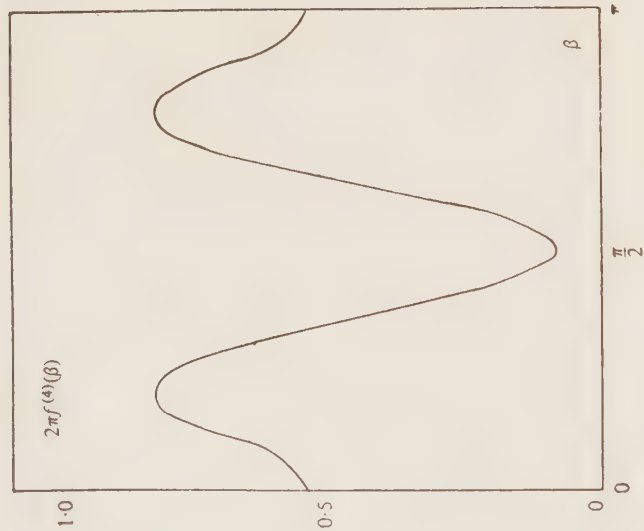


Fig. 4a.

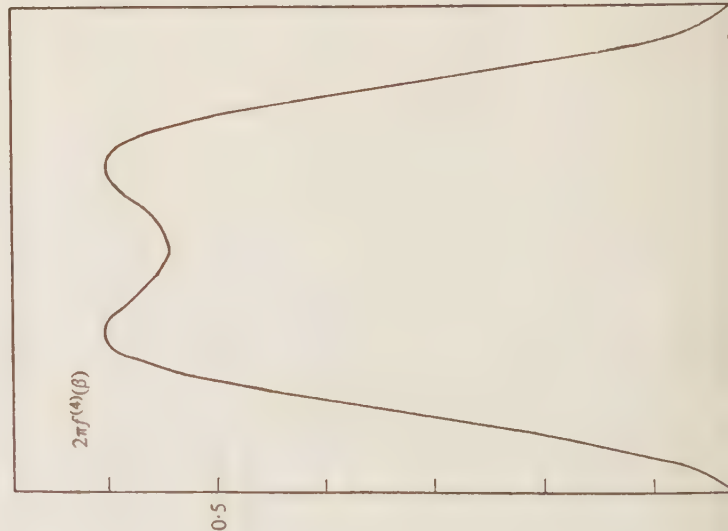


Fig. 4b.

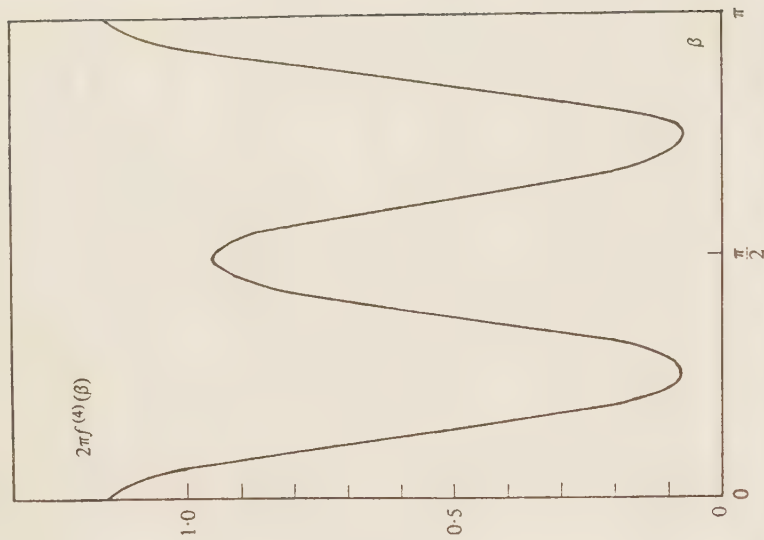


Fig. 4c.

Fig. 4. The distribution density function $f^{(4)}(\beta)$, calculated by using the first four non-vanishing elements of the power expansion

$$f(\beta) = \frac{1}{2\pi} \sum_{k=0}^{\infty} \frac{2k+1}{2} \langle D_{00}^{(k)}(\beta) \rangle D_{00}^{(k)}(\beta).$$

$\langle D_{00}^{(0)}(\beta) \rangle = 1$. (a) $\langle D_{00}^{(2)}(\beta) \rangle = 0.20$ and $\langle D_{00}^{(4)}(\beta) \rangle = -0.10$. (b) $\langle D_{00}^{(2)}(\beta) \rangle = -0.10$ and $\langle D_{00}^{(4)}(\beta) \rangle = 0.05$. (c) $\langle D_{00}^{(2)}(\beta) \rangle = -0.10$ and $\langle D_{00}^{(4)}(\beta) \rangle = 0.20$.

slightly favoured compared to being perpendicular to it. Often, $\langle D_{00}^{(2)}(\beta) \rangle$ is much larger than $\langle D_{00}^{(4)}(\beta) \rangle$ and the qualitative interpretation of order parameters gives no problem. Only for small negative-order parameters does one have to be careful.

IV. DETERMINATION OF MOLECULAR ORIENTATION

(A) Chromophore orientation in liquid crystals with polarized absorption spectroscopy

Quantitative information about the molecular orientation in an anisotropic liquid crystalline system or in a membrane can be obtained with polarized absorption spectroscopy only if the two following conditions

are fulfilled: (1) The sample studied must be macroscopically aligned or rather the orientational distribution density of the liquid crystalline *directors* or the membrane fragments must be known. (2) The direction of the transition moments in the molecular coordinate system of the chromophore must be determined and these transitions should also be easily identified in the absorption spectrum.

The macroscopical alignment of thermotropic liquid crystals can be accomplished in different ways, e.g. by a magnetic field or by putting the sample between two ordinary glass microscope slides which have been carefully rubbed lengthwise with for example paper (Brown, Doane & Neff, 1971). Usually, lyotropic mesophases are harder to align than thermotropics, but in many cases lamellar and hexagonal phases might align when pressed between glass plates (De Vries & Berendsen, 1969; Lindblom, 1972). Further, Lawson & Flautt (1968) discovered that some liquid crystalline systems on addition of salt spontaneously align in a magnetic field. Concerning the direction of transition moments, the knowledge is quite good for highly symmetric molecules, but unfortunately not so for most of the often low symmetrical biological chromophores. By using several spectroscopic techniques, however, we succeeded to determine the directions of the transition moments of the first three bands in the electronic absorption spectrum of flavin (Johansson *et al.* 1979, see Fig. 5). Although we are not going to discuss technical problems in this review a few important remarks will be given.

First, the best experimental accuracy is achieved for absorbances around unity of samples of good optical quality, i.e. no scattering to be noticed by eye. Secondly, certain care has to be taken when small order parameters are obtained especially if they are determined by using phase modulator lock-in techniques (Davidsson & Nordén, 1976*a, b*). By these techniques much smaller differences in absorbance can be registered in comparison with those measurable with the ordinary two spectra methods. Therefore for systems macroscopically aligned between plates the effects of multiple reflection must be taken into account especially for small order parameters (Johansson *et al.* 1978). The relative effect of multiple reflection (m) on the linear dichroism (LD) is illustrated in Fig. 6 for three different absorbances. Since the absorbance differences due to multiple reflexion normally is in the order of 10^{-3} the effect can usually be ignored when the two spectra method is used. A comparison between order parameters for some chromophores determined with both deuteron NMR and linear dichroism have also been

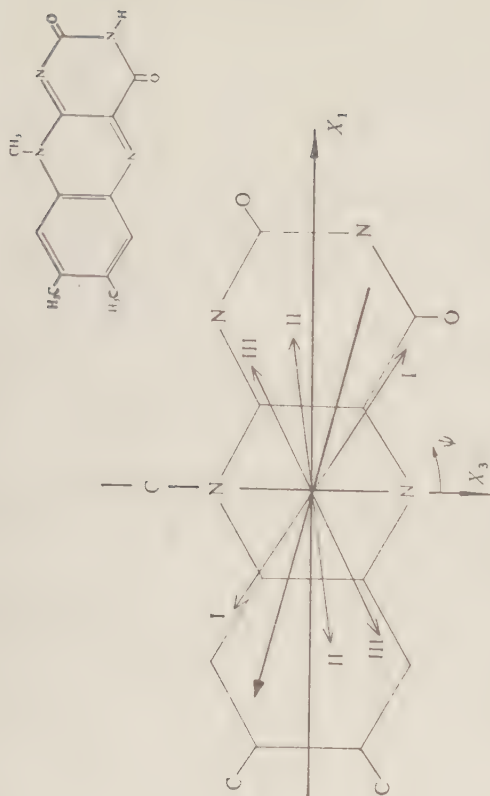
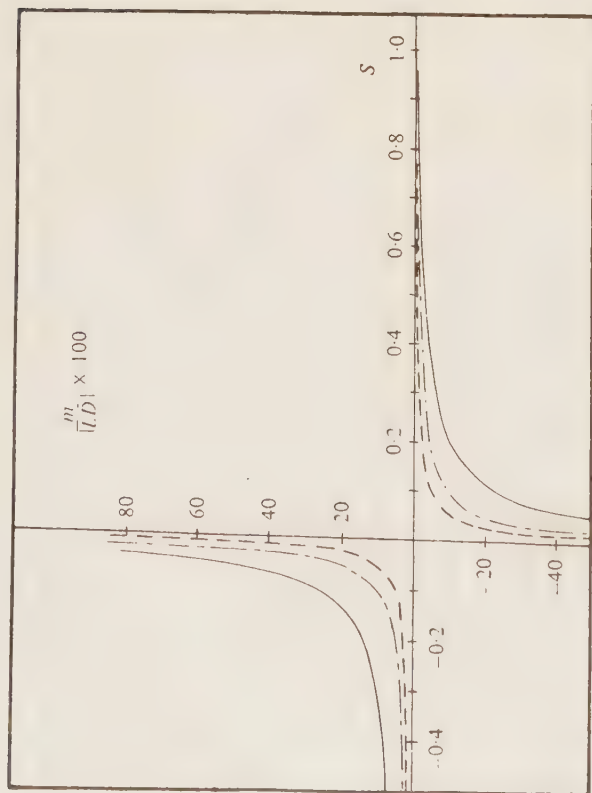


Fig. 5. The skeleton of the isalloxazine ring. The transition dipoles denoted I, II and III correspond to the absorption bands that occur at about 450, 350 and 270 nm respectively. Lumiflavine inserted. From Johansson *et al.* (1979).

reported (Johansson *et al.* 1978). Part of the results are shown in Table 1 and as can be seen there is a good agreement between the values obtained with the different methods. It is not quite obvious that the order parameters measured with both the methods should have the same value, since in the absorption experiment one measures the instant ensemble average value of the chromophore orientation while the NMR experiment measures the time average of the molecular orientation relative to the director. The observation of the same values with both methods thus means that on the NMR time scale, 10^{-4} s, the deuterated molecules have sufficient time to take on all the orientations in the ensemble. It can, however, be expected that the apparent order parameters will disagree for certain cases; for example, two-phase samples.

(1) Thermotropic liquid crystals

Most of the polarized spectroscopy on liquid crystals has hitherto been done on thermotropic systems (Sackmann & Möhwald, 1973; Priestly, Wojtowicz & Sheng, 1975). Sackmann & Möhwald (1973) determined the order parameter for a solute molecule in 'compensated' nematic mixtures of cholesteryl derivatives. They also pointed out that the prerequisite of transparency considerably limits the number of suitable liquid crystals for polarization studies of solutes since most of the known thermotropics



contain phenyl connected by unsaturated functional groups, which absorb up to the visible wavelength region. Besides these problems it should also be noted that the solute of interest to study has to be more or less hydrophobic to dissolve into the thermotropic liquid crystal. Therefore lyotropic liquid crystals should be preferable (see also below) where both hydrophilic and hydrophobic molecules can be solubilized (Johansson *et al.* 1979; Söderman *et al.*, 1980*a, b*). Furthermore, other absorbing groups of the liquid crystalline molecules can be easily avoided. Since it is interesting from a biological point of view to study the orientation of porphyrins (see IV (B)) the assignment of the electronic transitions is important and we will therefore briefly mention the works by Gale, Peacock & Samori (1976), Nördén & Davidsson (1976) and Fischer, Goldammer & Peltzl (1979). The latter authors found, in accordance with Gale *et al.* (1976), in their study of tetraphenylporphyrin incorporated in a thermotropic liquid crystal and in stretched polymers that the Soret and α bands are in-plane polarized $\pi^* \leftarrow \pi$ transitions. Thus neither of the strong transitions in the Soret region is a $\pi^* \leftarrow \pi$ transition as was previously proposed by Nördén and co-workers (1976).

* From Johansson *et al.* (1978).
† From Johansson *et al.* (1980).

Journeaux & Viovy (1978) investigated the orientation of chlorophylls in liquid crystals. Their paper is difficult to follow, since the authors do not define the various parameters used. The main result obtained was an order parameter of an unknown axis of rotation in the molecules (chlorophyll *a*, chlorophyll *b*, pheophytin *a* and methyl pheophorbide *a*). To be able to determine the direction of the transition moments in the molecule more than one physical method usually have to be used (Eaton *et al.* 1975; Johansson *et al.* 1979).

(2) Lyotropic liquid crystals

Steinemann, Stark & Luger (1972) have studied the orientation of chlorophyll in lipid bilayers. For these investigations they have developed a sensitive method for the measurements of the linear dichroism. A large part of the paper deals with the method and derivations of equations (although not general to all cases) used in the analysis of data. To the authors' knowledge this is the first work where linear dichroism of a tilted membrane has been obtained by rotating the electrical vector of the plane-polarized light and the observation of the signal by a phase-sensitive detector. It was found that the linear dichroism recorded at different orientations of the membrane with respect to the light beam varied linearly with $\cos^2 \omega$ (see also Fig. 7), i.e. the sample had uniaxial symmetry. Since the LD/A_1 did not vary with chlorophyll concentration up to a molar ratio between chlorophyll and lecithin of 0.7 it was concluded that the orientation of the transition moment corresponding to the wavelength $\lambda = 439$ nm was independent of the chlorophyll concentration in the membrane. The same was found to be true also for the $\lambda = 671$ nm transition. These authors also try to calculate an angle between the porphyrin plane of chlorophyll and the membrane. Although it was emphasized that it was assumed that *all* chlorophyll molecules in the bilayer had the same orientation such a calculation is useless since the order parameter is very small as shown in Table 2, where we have calculated *S* (from (2.18)) for some of the experimental data reported (Steinemann *et al.* 1972). Thus although the authors claim that the calculated values of the angle is not very sensitive to a small uncertainty of the orientation, a calculation of the angle cannot be done since the *distribution of the orientation* is unknown. Furthermore, it is remarkable to assume that *all* chlorophyll molecules have the same orientation in the membrane from a chemical point of view, since the chromophore most probably is located in the *water layer* (see Fig. 8). Note also that

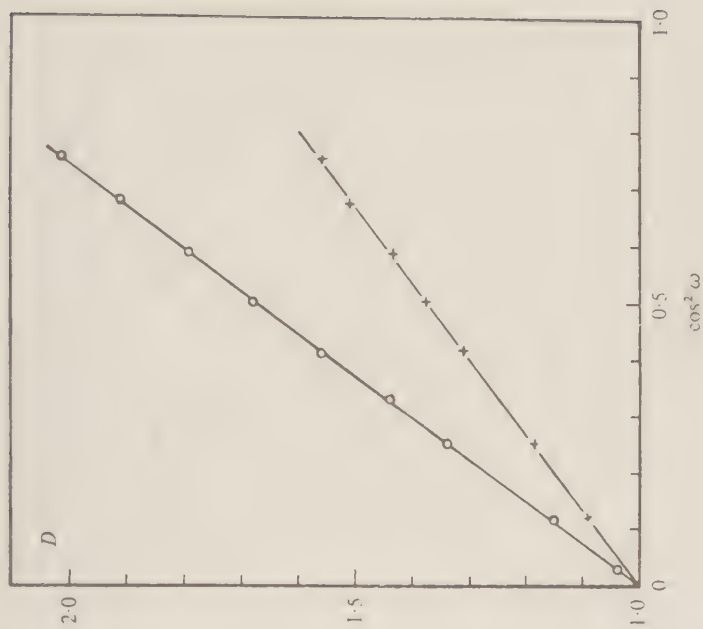


Fig. 7. The dichroic ratio, *D*, measured at different angles (ω) between the polarization vector of light and the normal of the quartz plates. The chromophore is retinal solubilized in the lamellar phases of sodium octanoate-decanol-water (17.8: 42.3: 39.9 %, w/w), denoted by $\times$, and sodium octanoate-octanoic acid-water (20.3: 34.5: 45.2 %, w/w) denoted by $\circ$.

order parameters have been calculated for long wavelengths only since it has been indicated (Goedheer, 1955; Gouterman, 1961; Weiss, Kobayashi & Gouterman, 1965) that the Soret band contains two transitions, polarized parallel and perpendicular to the transition moment giving absorption at long wavelength (i.e. 671 nm for chlorophyll *a*). Hlof (1974) has also studied the orientation of chlorophyll in lecithin bilayers. From his results shown in Fig. 9 we calculate from equation (2.17) an order parameter equal to 0.07 at $\lambda = 778$, which is in good agreement with those calculated from the data by Steinemann *et al.* (1972). The orientation of flavin mononucleotide (FMN) has been studied at various water concentrations in model membranes (Johansson *et al.* 1979). Four different order parameters were determined and it was found that all decreased strongly with increasing water content. This finding is to

TABLE 2. Order parameters (*S*) of chlorophyll *a* and *b* in different lipid bilayers obtained from linear dichroism (*LD*) and absorption (*A_⊥*) measurements

Lipid	Mole ratio	$LD/A_{\perp}^*$	<i>S</i> †
Dioleoyllecithin-Chl. <i>a</i>	0.18–0.70	0.04	0.05
Phosphatidylethanolamin-Chl. <i>a</i>	0.52	0.00	0.00
Phosphatidylserine-Chl. <i>a</i>	0.52	–0.02	–0.03
Dioleoyllecithin-Chl. <i>b</i>	0.4–1.2	0.22	0.22

* From Steinemann *et al.* (1972).

† Order parameter calculated using equation (2.18) corrected for refraction. The refractive index was assumed to be 1.5. $\omega = \pi/4$ and $\lambda = 671$ nm.

be expected if the FMN molecules are solubilized in the interface of the lipid bilayer and in the water layer. The orientation of retinal, on the other hand, did not vary appreciably in the lamellar phase of sodium octanoate-decanol-water or of the system sodium octanoate-octanoic acid-water (Johansson *et al.* 1980*b*). In this work the effect of one- and two-dimensional swelling of the multi-bilayer phase at various water contents was investigated. As can be seen from Fig. 10, the molecular order of retinal is almost constant at high water constants, where one-dimensional swelling occurs, i.e. addition of water in this part of the phase diagram will increase the amount of free water between the bilayers. Retinal is considered to be more or less anchored at the polar head group region through its hydrophilic aldehyde group.

Recently the binding and orientation of tetracine and procaine in model membranes were studied by measurements of LD (Johansson & Lindblom, 1980*c*). The chromophore binding can be explicitly included in the equation (2.18) as follows:

$$\frac{LD}{A_{\perp}} = \frac{3pS}{1-pS} \cdot n^2(1-n^2\cos^2\omega)^{-\frac{1}{2}}\cos^2\omega, \quad (4.1)$$

where *p* is the fraction of bound molecules in the anisotropic site. In equation (4.1) the difference in pathlength of the LD and *A_⊥* light beams and the refractive index, *n* of the aligned sample have been considered. The molecular structure of tetracine together with an experimental recording is shown in Fig. 11. For tetracine it was found that *LD/A_⊥* was independent of concentration of the drug in the bilayer, and it was concluded that *p* was close to unity. The uncharged form of tetracine was also studied and it was found that this form entered more

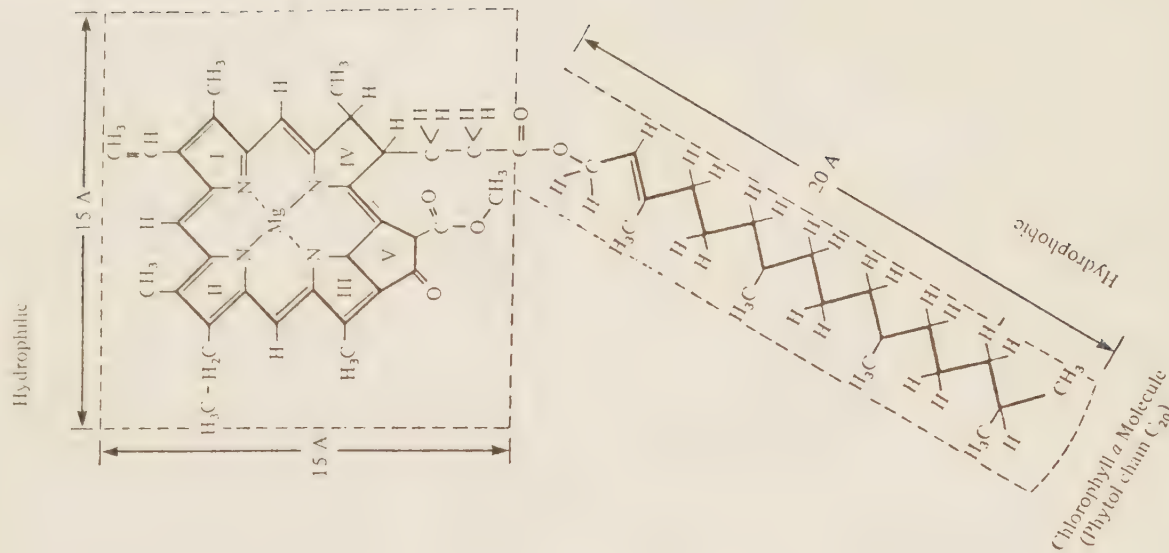


Fig. 8. Structure of the chlorophyll *a* molecule approximately to scale. The porphyrin head is approximately 15×15 Å and attached to the *C₂₀* phytol tail. The head is bent relative to the phytol group. The phytol tail is presumably embedded in the lipid matrix.

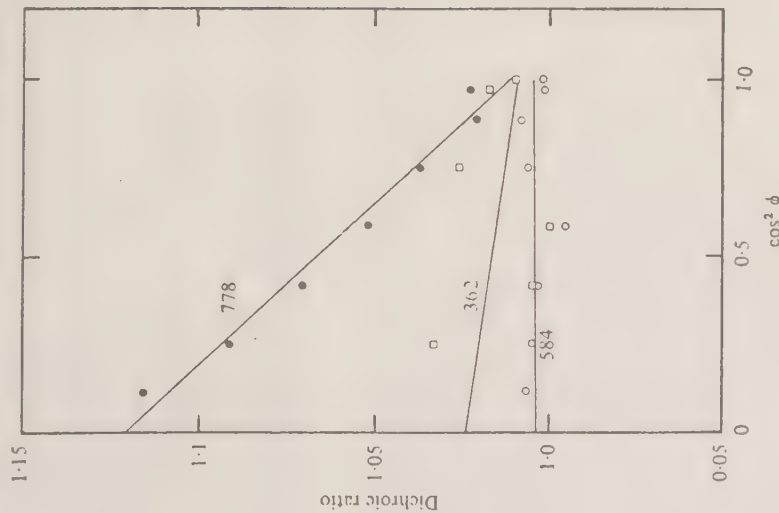


Fig. 9. The dichroic ratio of a bacteriochlorophyll-containing lecithin multilayer as a function of $\cos^2 \phi$, $\phi = (\pi/2) - \omega$. ●, 778 nm; ○, 584 nm; □, 362 nm. From Hoff (1974).

deeply into the lipid membrane. Procaine was shown to have a much lower order parameter $S \cong 0$. This drug is dissolved in the water region between the bilayers.

Polarized absorption spectroscopy has also been used together with deuteron NMR for a determination of the structure of a liquid crystalline phase that orients in a magnetic field (Söderman *et al.* 1980*a, b*).

Fromherz (1970) has investigated the energy transfer to cytochrome *c* in a model membrane. The samples were prepared by dipping a hydrophobic glass slide through a lipid protein film on a Langmuir trough (Bücher *et al.* 1967) whereby the film is transferred onto the slides. The lipid used was cadmium arachidate. By embedding the cytochrome protein at different distances from fluorescent dye molecules the transfer of excitation energy to cytochrome *c* was investigated (see

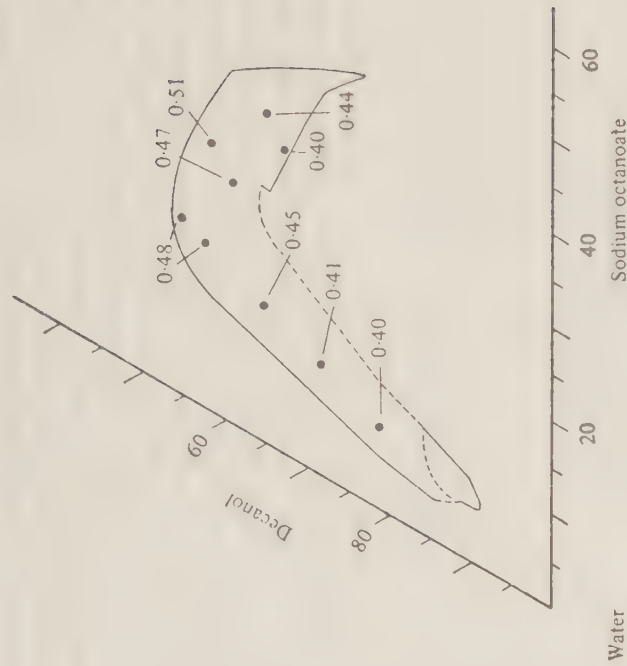


Fig. 10. Dependence of the order parameter of solubilized retinal on the composition for the sodium octanoate-decanol-water system. The dotted line indicates the uncertainty in the boundary of the lamellar phase.

Fig. 12). From the data presented an order parameter for the porphyrin ring of the protein can be calculated to be -0.12 , i.e. the ring plane tends to be orientated parallel to the membrane. An area of occupation at the membrane surface was calculated to be 770 Å^2 /protein molecule, indicating a closely packed layer of cytochrome *c* molecules.

(B) Proteins in biological membranes

The biological membrane constitute undoubtedly a very complex system, a fact which is reflected by the sparse literature on studies of protein orientation in membranes. Among the difficulties of such investigations by polarized light spectroscopy the two major ones are the problem of getting macroscopically aligned samples and that the recorded spectrum often exhibits overlapping signals from a range of chromophores in the intact membrane. In some favourable cases, however, the first problem might be circumvented by using time resolved fluorescence on non-aligned membranes as discussed in Section V. That considerable difficulties exist are demonstrated by the reports on the orientation of the

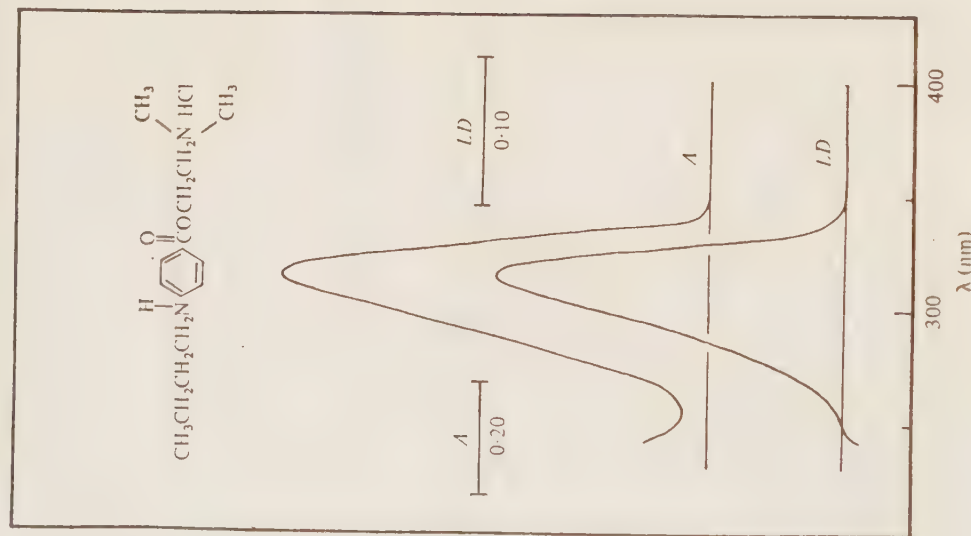


Fig. 11. The linear dichroism and absorption spectra of tetracaine in the lamellar phase of monoctanoïn-water (70 : 30 % w/w) at 22 °C. The structure formula shown is tetracaine.

heme groups in cytochrome oxidase, when different workers (Junge & DeVault, 1975; Erecińska, Wilson & Blasie, 1978) came to completely opposite conclusions. According to our opinion this shows that it is preferable to first study model systems like a lipid bilayer with an incorporated protein, before work on an intact membrane can be performed. Unfortunately, we have not found any such studies. Here the discussion will therefore be confined to a look at the apparent problems

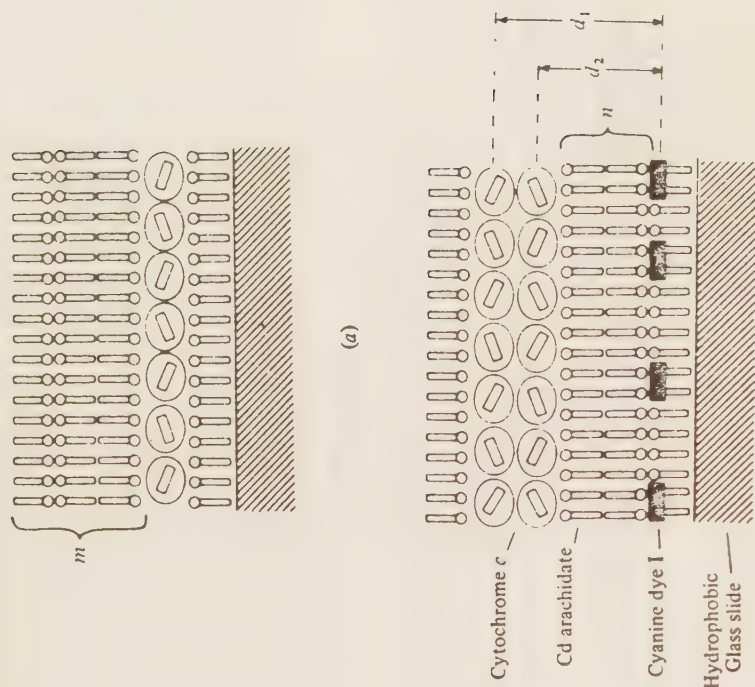


Fig. 12. Multilayer assemblies used for (a) measuring the absorbance of a cytochrome *c* layer; (b) investigating energy transfer from a cyanine dye to cytochrome *c*. From Fromherz (1970).

occurring in the works of polarized absorption on membranes containing proteins having porphyrin groups; especially haem and chlorophyll.

Erecińska and co-workers have reported several spectroscopic studies using polarized absorption, ESR and X-ray, to investigate the structure of cytochrome oxidase membrane systems (Erecińska, Blasie & Wilson, 1977; Erecińska, Wilson & Blasie, 1978; Blasie, Erecińska & Leigh, 1978). Their experimental findings from the polarized absorption studies are summarized in Table 3, together with order parameters of the haem plane calculated from equation (2.17), although the authors reported an angle between the haem plane and the director. Single-crystal polarized absorption studies of haemoproteins have shown the existence of an effective fourfold rotation axis of the haem (Eaton & Hochstrasser, 1967)

TABLE 3. Dichroic ratios, D , at 421 nm and 598 nm at different angles of incidence, $\omega = \pi/4$ and $\pi/6$, reported by Blasie *et al.* (1978): S is the calculated order parameter* of the haem plane

ω	$D(421 \text{ nm})$	$S^*(421 \text{ nm})$	$D(598 \text{ nm})$	$S^*(598 \text{ nm})$
45°	1.1	0.1	1.4–1.6	0.4–0.5
30°	1.2	0.1	1.5–2.0	0.2–0.4

* Equation (2.17) corrected for refraction. The refractive index was assumed to be 1.5.

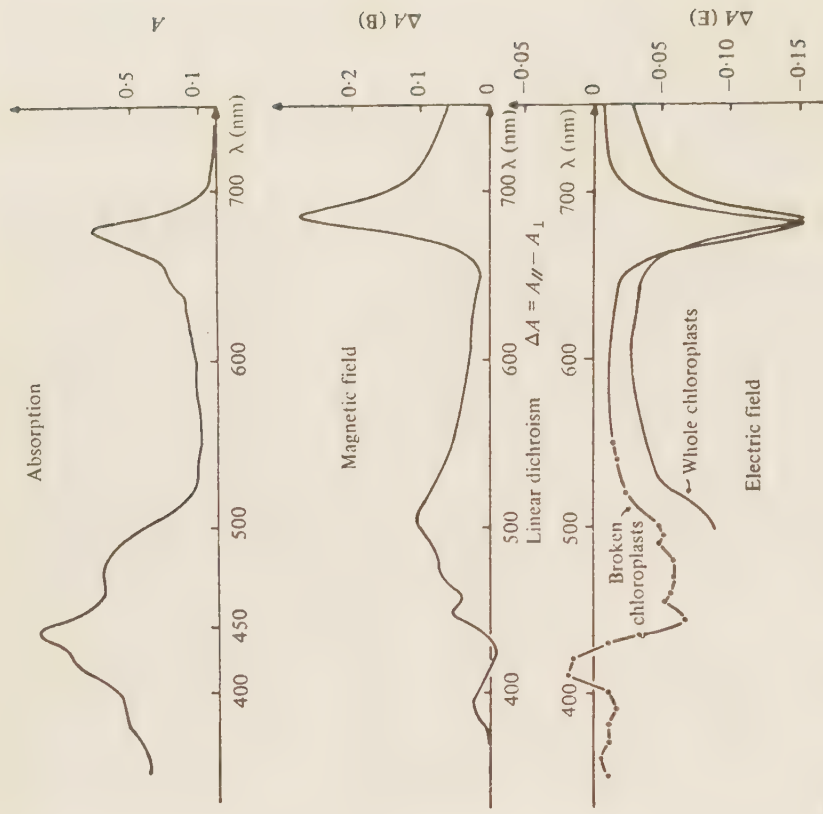
and this is also confirmed for cytochrome oxidase in solution (Junge & DeVault, 1975). Thus the order parameter of the *normal* to the haem plane obtained at the higher wavelength (the α -band) in the Table 3 will be less than $-\frac{1}{2}$. This is very intriguing, since the order parameter is limited to take the following values $-\frac{1}{2} \leq S \leq 1$. Furthermore, the S -value obtained from the Soret band is much smaller than that from the α -band, although both should describe the orientation of the haem plane. It is not easy to sort out these problems with the data available and many explanations can be given. However, since the order parameter obtained from the α -band vary with the angle between the membrane and the incident light, it is strongly indicated that the sample is not uniaxial (cf. Fig. 7, and app. A, equation A4), probably due to a non-perfect macroscopical alignment. Junge & DeVault (1975) tried to get information about the orientation of the a_3 haem of cytochrome oxidase by using a photoselection method applied on an isotropic suspension of mitochondrial membranes. The linear dichroism was observed after excitation with a flash of linearly polarized light. It is well known (Albrecht, 1961) that for an isotropic sample containing immobile chromophores having disklike symmetry ($D_{nh}; n \geq 3$) the dichroic ratio is equal to $4/3$ after such an excitation. This value was also obtained by Junge & DeVault (1975) for a suspension of membranes containing cytochrome oxidase. Therefore, they argued, if now the haemoprotein rotates within the membrane around an axis perpendicular to the membrane, then the haem plane must be orientated parallel to the membrane, i.e. $S = 1$ for the normal of the porphyrin ring (cf. also Table 3). Of course, in general, the order parameter cannot be obtained with this method, since it must always be equal to *unity*. On the other hand, it should be possible to modify the method according to the principles given in Section V. Also, the results by Junge & DeVault (1975) seem to us to be somewhat

unrealistic and it should be noted that the determination of a dichroic ratio of $4/3$ for these complicated systems demands an extremely good instrumentation. One of the major experimental difficulties is probably that of depletion, which to some extent has been discussed previously (Lessing & von Jena, 1976; von Jena & Lessing, 1979; Naqvi, 1980).

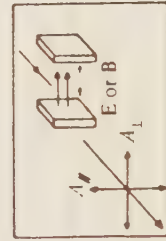
From the reports hitherto in the literature we must conclude that further studies are needed before an answer from polarized light spectroscopy can be given about the orientation of cytochrome oxidase in membranes. Maybe, an appropriate method for such an investigation can be based on a time resolved technique on an isotropic system (cf. Section V).

The orientation of chlorophyll in chloroplast membranes has been studied during many years by various workers. In this review we will only discuss some of the later reports. Breton, Michel-Villaz & Pailletin (1973) measured the linear dichroism directly by measuring the rotation of the plane of polarization of light transversing an anisotropic sample. Starting with this work Breton and co-workers have performed a systematic investigation of various methods of macroscopically alignment of whole or fractions of chloroplast membranes. In their first work three different techniques were used, namely (a) chloroplasts were spread with a paintbrush over an optically polished quartz plate, (b) a 'dry film' of membranes were made by air-drying a drop of suspension of isolated lamellae on a quartz plate, (c) membranes were aligned in a quartz cell by a magnetic field up to 1.05 T with the field being perpendicular to the light beam. It was found that the order parameters obtained for the three different methods were remarkably similar, being $S(a) = -0.08$, $S(b) = -0.17$ and $S(c) = -0.14$, all measurements at 681 nm. It is not surprising that the 'dry-film' gave the highest absolute value of the order parameter. At a higher magnetic field, however, the order parameter will approach a higher limiting value, since a stronger field eventually leads to a saturation of the orientation (Geacintov, Van Nostrand & Becker, 1971; Geacintov *et al.* 1972). The spreading and the 'dry-film' methods seem not to be useful for a quantitative determination of the molecular orientation of chlorophyll relative to the membrane surface, simply because the macroscopical alignment is not good enough. The magnetic field technique can be used only if the orientation of the chloroplast membrane in the magnetic field is known. The following arguments that the membranes tend to be orientated perpendicular to the magnetic field have also been reported (Becker *et al.* 1973). From the dichroic ratio

obtained for rhodamin B, with which the membranes were stained, it was concluded that the lamellae tended to be orientated perpendicular to the magnetic field. Becker *et al.* (1973) also discussed various causes of orientation. Lonsdale (1937) has shown that the diamagnetic susceptibility is greater along the carbon chain axis $|\chi_a|$ than perpendicular to it $|\chi_\perp|$. Therefore the lipid molecules should line up with their carbon chains perpendicular to the magnetic field (Hong, Mauzerall & Mauro, 1971), leading to a parallel alignment of the membranes along the magnetic field. This was not observed experimentally (Becker *et al.* 1973), and it can be concluded that the lipids cannot be responsible for the orientation of membranes in the magnetic field. On the other hand, the susceptibility normal to the porphyrin ring is much higher than within the plane (Lonsdale, 1937) and the porphyrin rings should therefore tend to be orientated parallel to the magnetic field. Becker *et al.* (1973) estimated that the chlorophyll molecules had sufficiently high anisotropic susceptibility to dominate the diamagnetic anisotropy of the chloroplast. However, this must be highly dependent on the distribution (order) parameter of the porphyrin rings and since the membrane have uniaxial symmetry the individual porphyrin susceptibilities will add up to effective $|\chi_a|$ (membrane) and $|\chi_\perp|$ (membrane), the latter being largest. This means, according to Becker *et al.* (1973), that the porphyrin ring tends to be orientated perpendicular to the membrane plane. However, from the experiment by Breton (1977) an order parameter of -0.2 can be calculated, i.e. the porphyrin ring tends to be orientated parallel to the membrane, given that the latter is orientated perpendicular to the magnetic field. Thus the alignment of a chloroplast membrane cannot be given such a simple explanation, and this was perhaps also to be expected. Note also that the orientation of the porphyrin ring is much larger than in a lipid bilayer (cf. IV, A 1). Recently, the linear dichroism $LD(E)$ of chloroplasts aligned by an electric field has also been investigated (Gagliano, Geacintov & Breton, 1977) and the data obtained were compared with $LD(B)$ from membranes orientated by a magnetic field (see Fig. 13). It was found that $LD(E)$ was negative while $LD(B)$ was positive and further that the ratio of the linear dichroisms was about -1.8 to -1.9 . Therefore it was concluded that the membranes were aligned parallel to the electric field as shown in Fig. 13 (theoretically for such a case the ratio $LD(B)/LD(E) = -2$).



(A) Magnetic field



(B) Electric field



(C) Chromophore orientation in liquid crystals from polarized emission spectroscopy

There are very few studies of the molecular orientation by the use of fluorescence. Badley, Martin & Schneider (1973) have studied a number of chromophores solubilized in lecithin lipid bilayers, and a review has been written on fluorescence techniques in the study of biological systems (Badley, 1976). Some reports have appeared where the orientation of a chromophore in thermotropic liquid crystals has been determined (Cehelnik *et al.* 1976; Dolganov & Bolotin, 1978; Chapoy & DuPré, 1979). The latter authors studied the orientation of 4-dimethylamino-4'-nitro-stilbene embedded in the liquid crystals p-methoxybenzlidene-p'-n-butylaniline (MBBA). By using their data we have calculated the distribution function $f^{(0)}(\beta)$ at three different temperatures (see Fig. 14). As can be seen from the figure both the order parameters $\langle D_{00}^{(2)}(\beta) \rangle$ and $\langle D_{00}^{(4)}(\beta) \rangle$ decreases with increasing temperature and $\langle D_{00}^{(4)}(\beta) \rangle$ also goes through zero. The influence of $\langle D_{00}^{(4)}(\beta) \rangle$ is here to lower the probability of finding the molecular z -axis (chosen along the coinciding transition moments) along the director, and to broaden the distribution.

V. TIME RESOLVED FLUORESCENCE OF MODEL MEMBRANE SYSTEMS

(A) Orientation and molecular motion

Generally the fluorophores will move during their lifetime in a solution or in a membrane system. A chromophore solubilized in a lipid bilayer system, like vesicles or multilamellar systems, will therefore undergo anisotropic rotational diffusion within the time scale of the fluorescence experiment, i.e. in the order of nano-seconds. Thus a molecule excited at a certain orientation in the bilayer will emit fluorescence at a different orientation and the fluorescence anisotropy, r , defined by equation (5.1) is time-dependent:

$$r(t) = \frac{F_{\parallel}(t) - F_{\perp}(t)}{F_{\parallel}(t) + 2F_{\perp}(t)}. \tag{5.1}$$

FIGURE 13

Fig. 13. Absorption spectrum (A) and linear dichroism spectra in magnetic ($\Delta A(B)$) and electric ($\Delta A(E)$) fields of whole and broken chloroplasts. The rectangular insert shows the directions of the electric (E) and magnetic (B) fields and the polarizations of light. The absorbances of the suspensions, $A = 0.6-0.7$ at 680 nm. (A) and (B) show the mode of orientation of the chloroplasts in magnetic and electric fields. The lamellar planes are represented by rectangular plates. From Gagliano *et al.* (1977).

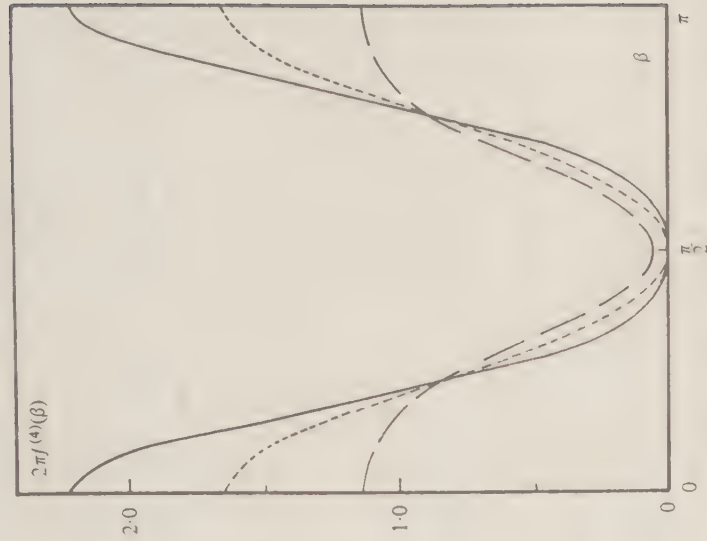


Fig. 14. Calculated distribution density functions of 4-dimethylamino-4'-nitrostilbene dissolved in the liquid crystalline phase of p-methoxybenzylidene-p'-n-butylaniline at three temperatures. The data used (from Chapoy & DuPré, 1979) are $\langle D_{00}^{(2)}(\beta) \rangle = 0.52$, $\langle D_{00}^{(4)}(\beta) \rangle = 0.09$ at 20 °C (—); $\langle D_{00}^{(2)}(\beta) \rangle = 0.43$, $\langle D_{00}^{(4)}(\beta) \rangle = 0.01$ at 30 °C (----), and $\langle D_{00}^{(2)}(\beta) \rangle = 0.32$, $\langle D_{00}^{(4)}(\beta) \rangle = -0.03$ at 37 °C (- -).

$F_{\parallel}(t)$ and $F_{\perp}(t)$ stand for the time-dependent fluorescence intensities detected with a linear polarizer set parallel and perpendicular to the direction of the excitation polarization. $r(t)$ is a convenient experimental quantity for macroscopically isotropic systems, e.g. vesicles, micellar solutions, membrane suspensions and cubic lyotropic liquid crystals. For a macroscopically aligned system the polarization, p , is a more practical experimental quantity as was pointed out in Section II (B). It has been shown previously (Soleillet, 1929; Perrin, 1936) that for fluorophores in a solvent

$$F_{\parallel}(t) - F_{\perp}(t) = \frac{2}{5} \left(\frac{3 \cos^2 \theta(t) - 1}{2} \right) \left(\frac{3 \cos^2 \delta - 1}{2} \right) kw(t) \\ = \frac{2}{5} D_{00}^{(2)}(\theta(t)) D_{00}^{(2)}(\delta) kw(t) \tag{5.2}$$

and

$$F_{\parallel}(t) + 2F_{\perp}(t) = kw(t), \tag{5.3}$$

where k is a constant and $w(t)$ denotes the probability of emission at the time, t , and $\theta(t)$ is the rotational angle of the emission transition moment between time zero and t , δ is the angle between the emission and the absorption moments. For a fluorophore solubilized in an aggregate like a vesicle or micelle, however, the rotational diffusion is anisotropic and $\theta(t)$ will depend on the orientation of the chromophore in the aggregate. This can be taken into account by evaluating the space average of equation (5.2) for a molecule having cylindrical symmetry around the absorption and/or the emission transition moment (cf. Appendix C)

$$F_{\parallel}(t) - F_{\perp}(t) = \frac{2}{3} \langle D_{00}^{(2)}(\theta(t)) \rangle D_{00}^{(2)}(\delta) k w(t). \quad (5.4)$$

For a fluorophore dissolved in a solvent it is, in general, assumed that $w(t)$ decays exponentially with time, and such a behaviour has also been shown by measurements of the total intensity (cf. (5.3)). For a chromophore in a solvent we therefore put

$$w(t) = \frac{1}{\tau} e^{-t/\tau}. \quad (5.5)$$

For a fluorophore being in a site with an anisotropic environment, however, the probability of emission may or may not show an exponential time dependence, since $w(t)$ may vary with the orientation of the fluorophore in the aggregate in question. Multiexponential decay curves have also been observed (Hildenbrand & Nicolau, 1979, and papers cited therein). The treatment of a system showing a nonexponential $w(t)$ is not trivial and will be outside the scope of this work and we will assume that the anisotropy has a negligible effect on $w(t)$. Then from equations (5.3) and (5.4) the following expression on $r(t)$ is obtained:

$$r(t) = \frac{2}{3} \langle D_{00}^{(2)}(\theta(t)) \rangle D_{00}^{(2)}(\delta). \quad (5.6)$$

On the time scale of the fluorescence experiment the time dependence of $r(t)$ is entirely determined by the anisotropic motion of the molecules *within* the aggregates. This means that $r(t)$ will depend on the molecular orientation in the aggregate and within the lifetime of the fluorophores r will never approach zero. Thus $r(t)$ contains information about both motion and orientation of the chromophores. The molecular motion is described by the orientational correlation function, which can be obtained from $\langle D_{00}^{(2)}(\theta(t)) \rangle$ in equation (5.6) by applying the closure theorem of Wigner rotation matrix elements (Brink & Satchler, 1968):

$$\langle D_{00}^{(2)}(\theta(t)) \rangle = \sum_{m=-2}^2 \langle D_{m0}^{(2)}(\Omega_0) \rangle D_{m0}^{(2)*}(\Omega), \quad (5.7)$$

where Ω and Ω_0 represent the orientation of the transition moment relative to the local director of the aggregate at time t (emission) and zero (excitation), respectively. In the diffusion limit

$$\langle D_{m0}^{(2)}(\Omega_0) \rangle D_{m0}^{(2)*}(\Omega) = \int_{\Omega_0 \Omega} f(\Omega_0) D_{m0}^{(2)}(\Omega_0) g(\Omega_0 | \Omega, t) D_{m0}^{(2)*}(\Omega) d\Omega_0 d\Omega. \quad (5.8)$$

Here $f(\Omega_0)$ is the uniaxial distribution density describing the molecular orientation relative to the normal of the surface of the spherical or the lamellar aggregate. $g(\Omega_0 | \Omega, t)$ is the conditional probability density that if at time zero the molecule was orientated at Ω_0 , then at a time t it will be at Ω . The conditional distribution density must satisfy the initial condition

$$g = (\Omega_0 | \Omega_0) \delta(\Omega_0 - \Omega), \quad (5.9)$$

where $\delta(\Omega_0 - \Omega)$ represents the Dirac delta function. Further at sufficiently long time (t_{∞}) the excited molecules have taken all possible orientations of the orientational distribution density function $f(\Omega)$ and we can write

$$g(\Omega_0 | \Omega, t_{\infty}) = f(\Omega), \quad (5.10)$$

which is always true, unless the excited molecules are different (charge distribution, shape, etc.) from the ground state molecules. Equations (5.6) and (5.7) then give

$$r(0) = \frac{2}{3} D_{00}^{(2)}(\delta) \quad (5.11)$$

and the equations (5.6) and (5.10) yield

$$r(t_{\infty}) = \frac{2}{3} \langle D_{00}^{(2)}(\Omega) \rangle^2 D_{00}^{(2)}(\delta) \equiv \frac{2}{3} S^2 D_{00}^{(2)}(\delta). \quad (5.12)$$

Equation (5.11) has been given previously in the literature (Kinosita, Kawato & Ikegami, 1977; Heyn, 1979; Jähnig, 1979*b*). This is a most interesting result, telling us that it is thus possible to determine the order parameter of a chromophore solubilized in a spherical aggregate of an isotropic solution, if the angle between the absorption and emission transition moments are known. The order parameter describes the average orientation of the cylindrical symmetry axis in the molecule relative to the director. It should be noticed, however, that the sign of the order parameter cannot be obtained unless $|S| > 0.5$, when it must be positive. Further if $\delta = 54.7^\circ$, the so-called magic angle, then $D_{00}^{(2)}(\delta) = 0$, implying that $r(t) = 0$ and no information can be obtained. The order parameter will also strongly depend on the *aggregate shape*, since the director is determined by the symmetry axis of the aggregate. A fluorophore solubilized in a spherical micelle should therefore give an apparent S -value which is twice as large as for a rodlike micelle. This fact can be utilized

for structural investigations of amphiphile aggregates in isotropic systems (micellar solutions, cubic mesophases, microemulsions, etc.). In order to express the time-dependence of r explicitly, $g(\Omega_0|\Omega, t)$ must be obtained. This is a very complicated problem for an anisotropic system and only approximative expressions of $g(\Omega_0|\Omega, t)$ have been derived. Luckhurst, Setaka & Zannoni (1974) and Nordio & Busolin (1971) investigated this problem considering the rotational motion as a stationary Markov process, described through an anisotropic rotational diffusion equation. Their results predict a time-dependence of $r(t)$ containing infinitely many exponentials. This was also pointed out by Wennerström (1980) from entirely symmetry considerations of the correlation function. Thus it is a formidable task to get quantitative information about the rotational motion in an anisotropic system like a membrane from a measurement of $r(t)$. Therefore a phenomenological approach is usually taken (Kawato, Kinoshita & Ikegami, 1977; Lakowicz, Prendergast & Hogen, 1979) and r can be written

$$r(t) = [r(0) - r(t_\infty)] e^{-t/\phi} + r(t_\infty), \quad (5.13)$$

since it has been shown that experimental data in many cases, can be fitted to a one-exponential curve at short times. Of course one can include several exponentials in equation (5.12). ϕ here defines some kind of a relaxation time, characterizing the anisotropic rotational motion. Hopefully, it will be possible to make molecular models explaining ϕ for particular problems encountered in the future.

Many experiments using polarized fluorescence on anisotropic systems have been performed with the steady-state method (Shinitzky & Barenholz, 1978). Also in such a measurement r (steady state), r_s , will depend on both molecular ordering and molecular motion of the chromophore. This is easily seen from the following integration

$$r_s = \int_0^\infty r(t) \frac{1}{\tau} e^{-t/\tau} dt, \quad (5.14)$$

$$\text{giving} \quad r_s = r(t_\infty) + \frac{r(0) - r(t_\infty)}{1 + (\tau/\phi)}. \quad (5.15)$$

Obviously information about molecular ordering and/or dynamics is not easily obtained from r_s unless we know either τ/ϕ or $r(t_\infty)$. Note also that if $\tau/\phi \gg 1$, then the order parameter can be obtained from a steady-state experiment, further if $r_s = \frac{2}{3}$ then $\tau/\phi \ll 1$ and the molecules are immobile.

Time resolved fluorescence can also be exploited for the determination of order parameters of chromophores in *macroscopically anisotropic systems*. The basic principles of such a method are delineated below.

For a system in which the fluorophores are mobile during the life time of the excited state the elements $\langle F_{ij\omega} \rangle$ and $\langle F_{ij\perp} \rangle$ (cf. equations 2.22 and 2.23) become time dependent.

$$\langle F_{ij\omega}(t) \rangle = \int_{\Omega_0\Omega} [P_{XX}^L(\Omega_0) \sin^2 \omega + P_{ZZ}^L(\Omega_0) \cos^2 \omega - P_{XZ}^L(\Omega_0) \sin 2\omega] Q_{ij}^L(\Omega) f(\Omega_0) g(\Omega_0|\Omega, t) d\Omega_0 d\Omega, \quad (5.16)$$

$$\langle F_{ij\omega\perp}(t) \rangle = \int_{\Omega_0\Omega} P_{YZ}^L(\Omega_0) Q_{ij}^L(\Omega) f(\Omega_0) g(\Omega_0|\Omega, t) d\Omega_0 d\Omega. \quad (5.17)$$

Using equation (5.10) and assuming uniaxial symmetry

$$\langle F_{ij\omega}(t_\infty) \rangle = \langle P_{XX}^L \sin^2 \omega + P_{ZZ}^L \cos^2 \omega \rangle \langle Q_{ij}^L \rangle, \quad (5.18)$$

$$\langle F_{ij\perp}(t_\infty) \rangle = \langle P_{YZ}^L \rangle \langle Q_{ij}^L \rangle. \quad (5.19)$$

By determining the fluorescence intensities $F_\omega(t_\infty)$ and $F_\perp(t_\infty)$ with the polarization of the exciting light at an angle ω or $90^\circ(\perp)$ relative to the director the dichroic ratio (cf. 2.15) is given by

$$D = \frac{F_\omega(t_\infty)}{F_\perp(t_\infty)} = 1 + \frac{3 \sum_n \langle D_{on}^{(2)*} \rangle P_n^M \cos^2 \omega}{\text{Tr} \tilde{P} - \sum_n \langle D_{on}^{(2)*} \rangle P_n^M}. \quad (5.18)$$

Alternatively the transmission axis of the analyser can be set at the angles ω and $90^\circ(\perp)$ relative to the director. The polarization of the exciting light can be chosen arbitrary.

(B) The effect of cholesterol in lipid bilayers

The role played by cholesterol in membranes has been studied for many years using a variety of spectroscopic techniques; NMR (Mantsch, Saito & Smith, 1977), ESR (Smith & Butler, 1976) and polarized fluorescence (Cogan *et al.* 1973; Kawato, Kinoshita & Ikegami, 1978). Concerning the polarized fluorescence investigations a chromophoric probe molecule with high molar absorptivity and high quantum yield should be used. A frequently used hydrophobic probe for model membranes studies is all-trans-1,6-diphenyl-1,3,5-hexatriene (DPH), which also has been reported to exhibit a single exponential emission decay curve in solvents (Cehelnik *et al.* 1975). Other fluorophores that can be used for various purposes have been reported (Badley, 1976; Waggoner & Stryer, 1970).

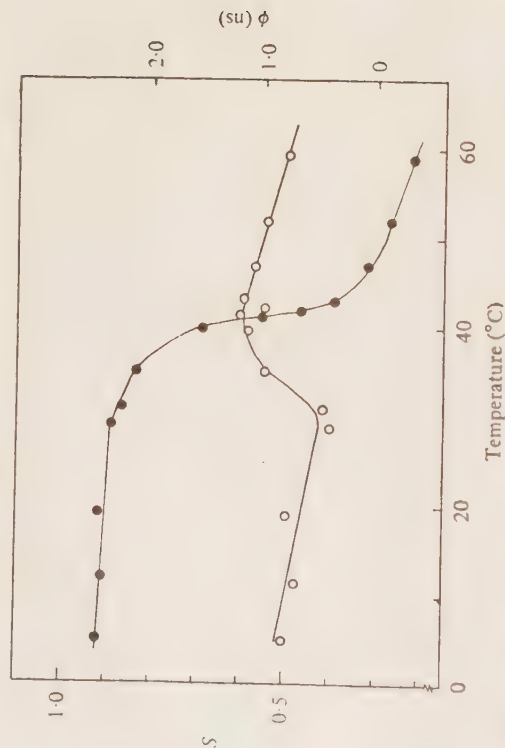


Fig. 15. The temperature dependence of the order parameter S (●) and the rotation relaxation time ϕ (○) in DPL vesicles. S has been calculated and ϕ taken from data obtained by Kawato *et al.* (1978).

Kawato *et al.* (1977) have studied the time resolved fluorescence of DPH solubilized in liposomes of dipalmitoyllecithin (DPL) at different temperatures. From their data an order parameter has been calculated from equation (5.12) assuming that $r_0 = 0.4$. Fig. 15, shows S as a function of temperature and as can be seen S approaches unity in the gel phase (crystalline) and then decreases to a much lower value in the liquid crystalline phase. It can thus be concluded that DPH is highly orientated in the gel phase, having its long axis almost perpendicular to the membrane surface, and that in the liquid crystalline phase DPH tends to be similarly but less orientated. The transition temperature obtained was 41°C , in agreement with previous determinations (Chapman, Williams & Ladbroke, 1967). Similar results for a number of lecithins, dimyristoyllecithin (DML), distearoyl lecithin (DSL) and dioleoyl lecithin (DOL) have been reported (Lakowicz *et al.* 1979).

The effect of cholesterol on the order parameter of DPH in lipid bilayers has been calculated from literature data (Kawato *et al.* 1978; Hildenbrand & Nicolau, 1979) using (5.12). Fig. 16 shows the results obtained, together with order parameters determined from deuterium NMR of perdeuterated stearic acid, incorporated as a probe in DPL and egg yolk lecithin (EYL) bilayers (Mantsch *et al.* 1977). It can be inferred from Fig. 16 that the order parameters obtained from both NMR and

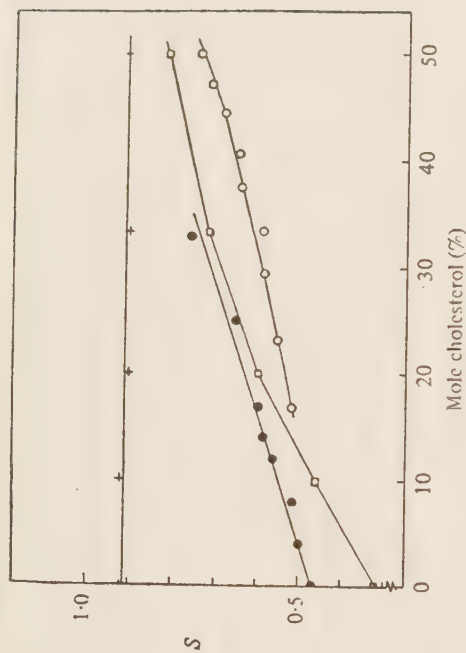


Fig. 16. The effect of cholesterol on the order parameter S of DPH and stearic acid solubilized in two lecithins. S refer to the long axis of DPH and to the 10 first carbon atoms of stearic acid. +, DPH in DPL vesicles at 10.5°C , □, DPH in DPL vesicles at 49.5°C (calculated from Kawato *et al.* 1978); ○, DPH in EYL vesicles at 25°C (calculated from Hildenbrand & Nicolau, 1979); ●, stearic acid in the lamellar phase of EYL at 23°C (Mantsch *et al.* 1977).

polarized fluorescence increases with increasing cholesterol content. However, the average orientation of DPH is slightly lower than for the first ten methylene groups of the carbon chain of the acid. The order parameter of DPH is about 0.9 for a lipid sample below the phase transition temperature, T_m , from gel to liquid crystal. That the order parameter differs from unity here is probably due to the fact that the perturbation of the probe cannot be neglected. Addition of cholesterol, up to 50 mole %, to a sample below T_m does not affect the order parameter (cf. also Fig. 17). This behaviour is expected since cholesterol has two effects here. First, the presence of cholesterol removes the gel to liquid crystalline phase transition as shown by differential scanning calorimetry (Chapman *et al.* 1967). Further the NMR band shape of the phospholipid-cholesterol-water mixture, far below the phase transition of the pure lipid-water system, is the same as that characteristic ('super-lorentzian') for liquid crystalline samples (Ulmius *et al.* 1975; Wennerström & Lindblom, 1977). Second, it is well known that cholesterol increases the molecular ordering in the liquid crystalline phase (Mantsch *et al.* 1977). Thus, although cholesterol has the effect of inducing the liquid crystalline state below the transition temperature, the molecular ordering is not decreased but is counteracted by the second effect.

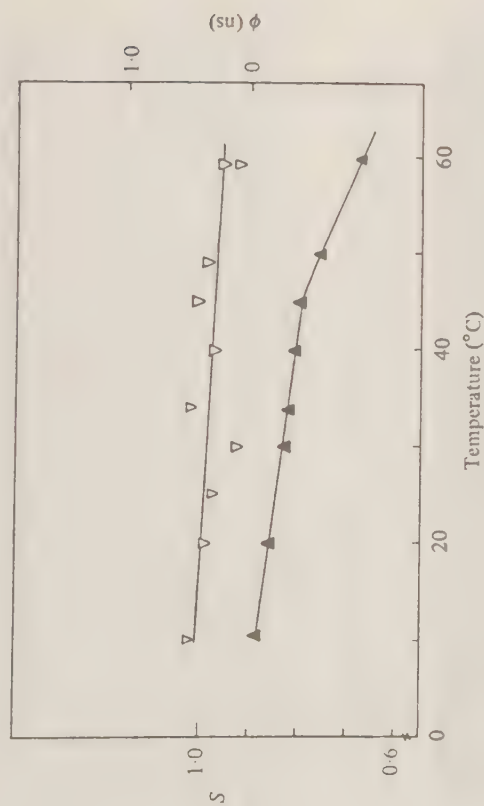


Fig. 17. The temperature dependence of the order parameter S (▲) and the rotation relaxation time ϕ (▽) in DPL vesicles containing 33 mole % cholesterol. S has been calculated and ϕ taken from data obtained by Kawato *et al.* (1978).

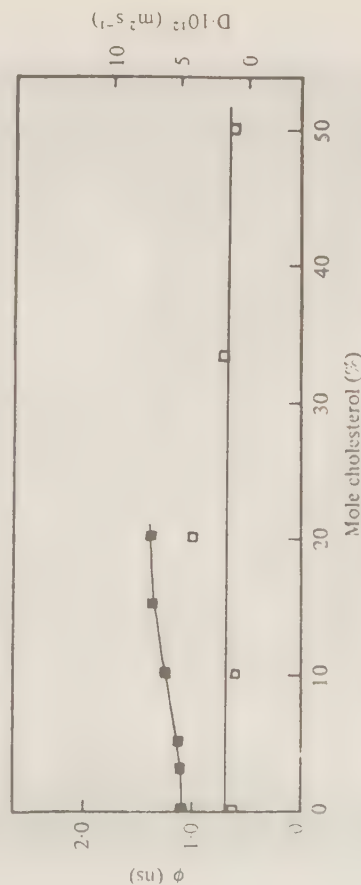


Fig. 18. The effect of cholesterol on the rotation relaxation time of DPH in DPL at 49.5 °C (□). (Kawato *et al.* 1978) and the lipid lateral translational diffusion of DOL at 35 °C (■) (Lindblom *et al.* 1980).

Fig. 18 shows the dependence of the relaxation time, ϕ , on the cholesterol content in DPL bilayers. It is obvious from Fig. 18 that ϕ stays almost constant when the cholesterol content is varied. An unambiguous interpretation of ϕ in terms of molecular rotational diffusion coefficients is hard to make (cf. V. 1). However, from a 'wobbling in a cone' model (Kinosita, Kawato & Ikegami, 1977), Kawato *et al.* (1978)

calculated a diffusion coefficient of the long axis of DPH. Recently, the lateral diffusion coefficient, D , of different phospholipids in bilayers were determined with a NMR spin-echo technique (Lindblom *et al.* 1976c; Lindblom & Wennerström, 1976d, 1977; Lindblom, Johansson & Arvidson, 1980). It was found (Lindblom *et al.* 1980) that cholesterol slightly increases the translational diffusion of DOL in a bilayer (see Fig. 18). To summarize: both NMR and polarized fluorescence show that the effect of cholesterol is to increase the molecular ordering (S) of the hydrocarbon chains but that the molecular dynamics (ϕ , D_L) is almost unaffected. Recently, it was also shown that it is the structural or geometrical properties of cholesterol that is of great importance in biological membranes (Wieslander *et al.* 1980; Khan *et al.* 1980). Here it was found that cholesterol strongly influenced the lipid bilayer stability of membranes from *Acholeplasma laidlawii*.

(C) Other time resolved techniques

Protein rotational diffusion in membranes occur on a time scale of μs to ms. Hence, polarized fluorescence cannot be used to study the rotational motion of membrane proteins, because the fluorescent emission will decay before any detectable rotation can occur. Recently, several methods of investigating slow rotational diffusion have been developed. All these techniques have a common denominator, namely the exploiting of the long lifetime of the triplet state of the chromophore. The most frequently utilized method is based on a flash photolysis apparatus to detect the dichroism of absorption transients induced by a short pulse of linearly polarized light. A recent review of the application of this technique to membranes has been written by Cherry (1978). Relatively few studies of proteins incorporated in lipid bilayers have been done. Vaz, Austin & Vogel (1979) studied the triplet absorbance anisotropy of the integral membrane protein cytochrome b_5 incorporated into model membranes composed of dimyristoyllecithin. This investigation was feasible by using a derivative of cytochrome b_5 in which the haem group was replaced by the haem analogue, rhodium (III)-protoporphyrin ix. The experimental data were analysed according to the 'wobbling in a cone'-model (Kinosita *et al.* 1977) and the rotational diffusion constant was found to be $2.4 \times 10^5 \text{ s}^{-1}$ at 35 °C and $1.1 \times 10^4 \text{ s}^{-1}$ at 10 °C. The protein orientation was found to be high with $r(t_\infty)/r(0) = 0.6$ giving an order parameter of about 0.8.

By measuring the time resolved phosphorescence instead of the triplet

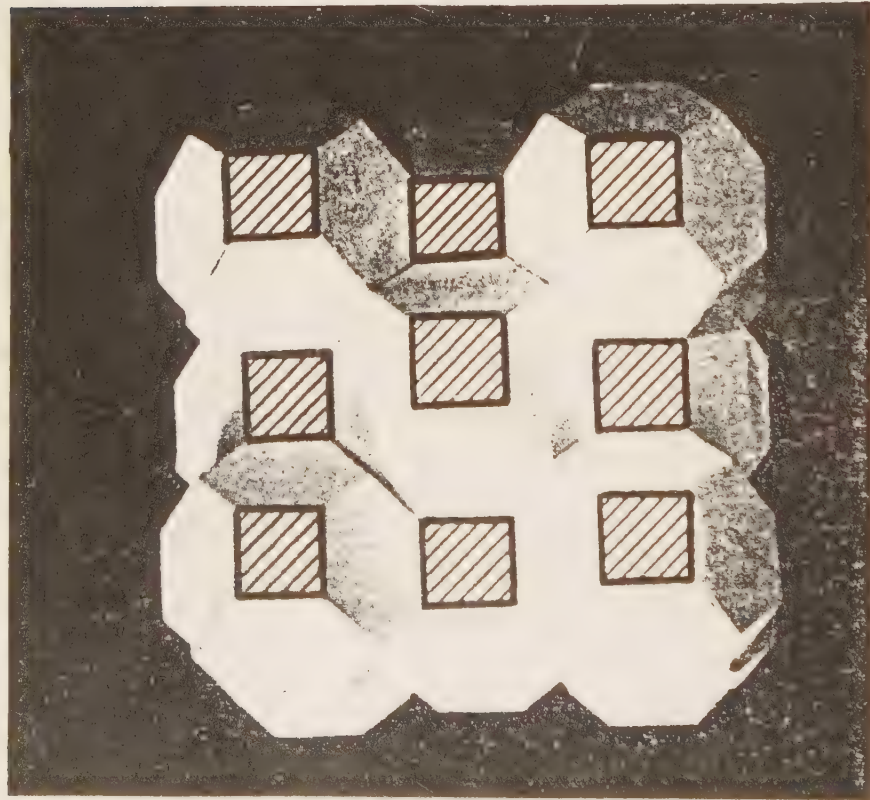


Fig. 19. The proposed structure of the cubic phase composed of 'open' tetra- and hexagonal-shaped surfaces. The hexagonal-shaped surfaces represent the lipid bilayer units and the hatched areas the water channels. From Lindblom *et al.* (1979).

absorbance the sensitivity can be much enhanced. In a recent work by Austin, Chan & Jovin (1979) an apparatus for measurements of the time resolved phosphorescence anisotropy has been constructed. These authors used this method to study the rotational diffusion of concanavalin A receptors of viable Friend erythroleukemia cells and of the band-3 anion transport system of human erythrocytes. It was shown that measurements could be done with probe concentrations (eosin) as low as 20 nM. Another possibility to extend the range of the time-scale is to study the depolarization of *E*-type delayed fluorescence (Naqvi & Wild, 1975). This method has been utilized by Greinert *et al.* (1979) to study

the rotational motion of eosin-labelled cytochrome P-450 incorporated into phospholipid vesicle.

A problem that may appear in these studies of protein rotation at such a long time-scale as ms is that the vesicle rotation itself will influence the experimental data. This problem can be circumvented by using a model membrane system like a cubic liquid crystalline phase, which is optically isotropic. Recently we showed (Lindblom *et al.* 1979) that the cubic phase of monolein and water is built up of lipid bilayer units (see Fig. 19) and could therefore serve as a good model membrane system. High amounts of lecithin can also be incorporated in this cubic phase (Tardell, Lindblom & Arvidson, unpublished data).

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APPENDIX A

The second-order irreducible tensor components are related to the cartesian second-order components (Hubbard, 1969) as:

$$\begin{aligned} P_{\pm 2} &= \sqrt{\frac{6}{4}}(P_{XX} - P_{YY} \pm 2iP_{XY}), \\ P_{\pm 1} &= \mp \sqrt{\frac{3}{2}}(P_{XZ} \pm iP_{YZ}), \\ P_0 &= \frac{1}{2}(3P_{ZZ} - \text{Tr } \hat{P}), \end{aligned} \quad (\text{A } 1)$$

The relation between Saupe's ordering matrix elements S_{ij} (Saupe, 1964) and the order parameters $\langle D_{0n}^{(2)*} \rangle$:

$$\begin{aligned} S_{xx} - S_{yy} &= \frac{2}{3} \langle D_{02}^{(2)*}(\beta\gamma) + D_{0-2}^{(2)*}(\beta\gamma) \rangle, \\ S_{zz} &= \langle D_{00}^{(2)}(\beta) \rangle = \frac{1}{2} \langle 3 \cos^2 \beta - 1 \rangle, \\ S_{xy} &= i \sqrt{\frac{3}{2}} \langle D_{02}^{(2)*}(\beta\gamma) - D_{0-2}^{(2)*}(\beta\gamma) \rangle = - \langle \frac{3}{2} \sin^2 \beta \sin \gamma \cos \gamma \rangle, \\ S_{xz} &= \sqrt{\frac{3}{2}} \langle D_{01}^{(2)*}(\beta\gamma) - D_{01}^{(2)*}(\beta\gamma) \rangle = - \langle \frac{3}{2} \sin \beta \cos \beta \cos \gamma \rangle, \\ S_{yz} &= i \sqrt{\frac{3}{2}} \langle D_{01}^{(2)*}(\beta\gamma) + D_{0-1}^{(2)*}(\beta\gamma) \rangle = \langle \frac{3}{2} \sin \beta \cos \beta \sin \gamma \rangle. \end{aligned}$$

Further, $S_{xx} = \frac{1}{2} \langle 3 \sin^2 \beta \cos^2 \gamma - 1 \rangle$
and $S_{yy} = \frac{1}{2} \langle 3 \sin^2 \beta \sin^2 \gamma - 1 \rangle.$

beneath. The molecular z -axis is here chosen parallel to the emission dipole moment.

$$\left. \begin{aligned} \langle A_{\perp} Q_{XX}^L \rangle &= \frac{1}{6} q^2 \text{Tr} \tilde{P} \langle 1 - D_{00}^{(2)}(\beta) \rangle + \frac{1}{6} q^2 \sum_m a_m P_m^M, \\ \langle A_{\perp} Q_{YY}^L \rangle &= \frac{1}{6} q^2 \text{Tr} \tilde{P} \langle 1 - D_{00}^{(2)}(\beta) \rangle + \frac{1}{6} q^2 \sum_m b_m P_m^M, \\ \langle A_{\perp} Q_{ZZ}^L \rangle &= \frac{1}{6} q^2 \text{Tr} \tilde{P} \langle 1 + 2D_{00}^{(2)}(\beta) \rangle + \frac{1}{6} q^2 \sum_m c_m P_m^M, \\ \langle A_{\perp} Q_{XY}^L \rangle &= \langle A_{\perp} Q_{XZ}^L \rangle = \langle A_{\perp} Q_{YZ}^L \rangle = 0. \end{aligned} \right\} \quad (\text{B } 1)$$

The coefficients a_m , b_m and c_m are listed in the table.

$$\left. \begin{aligned} \langle A_{\omega} Q_{XX}^L \rangle &= \frac{1}{6} q^2 \text{Tr} \tilde{P} \langle 1 - D_{00}^{(2)}(\beta) \rangle + \frac{1}{6} q^2 \sum_m \{b_m \sin^2 \omega \\ &\quad + (c_m + 2\langle D_{0m}^{(2)*}(\beta) \rangle \cos^2 \omega)\} P_m^M, \\ \langle A_{\omega} Q_{YY}^L \rangle &= \frac{1}{6} q^2 \text{Tr} \tilde{P} \langle 1 - D_{00}^{(2)}(\beta) \rangle + \frac{1}{6} q^2 \sum_m \{a_m \sin^2 \omega \\ &\quad + (c_m + 2\langle D_{0m}^{(2)*}(\beta) \rangle \cos^2 \omega)\} P_m^M, \\ \langle A_{\omega} Q_{ZZ}^L \rangle &= \frac{1}{6} q^2 \text{Tr} \tilde{P} \langle 1 + 2D_{00}^{(2)}(\beta) \rangle + \frac{1}{6} q^2 \sum_m (c_m \sin^2 \omega \\ &\quad - 2c_m \cos^2 \omega) P_m^M, \\ \langle A_{\omega} Q_{XY}^L \rangle &= \frac{1}{6} q^2 \sum_m d_m P_m^M \sin 2\omega \\ \langle A_{\omega} Q_{XZ}^L \rangle &= \langle A_{\omega} Q_{YZ}^L \rangle = 0. \end{aligned} \right\} \quad (\text{B } 2)$$

The coefficients $A_m, B_m, \dots, G_m$ used in equation (2.26) are related to a_m, b_m and c_m as follows:

$$\begin{aligned} A_m &= b_m - a_m; & B_m &= c_m - a_m; \\ C_m &= -3c_m - 2\langle D_{0m}^{(2)}(\beta)^* \rangle; & D_m &= a_m + b_m; \\ E_m &= 2c_m + 4\langle D_{0m}^{(2)}(\beta)^* \rangle; & F_m &= a_m + c_m; \\ G_m &= 2\langle D_{0m}^{(2)*}(\beta) \rangle - c_m. \end{aligned}$$

APPENDIX C

Fluorescence from molecules undergoing anisotropic rotational motion in a macroscopically isotropic system (micellar solution, etc.). On the basis of equations 2.20-2.21,

$$\langle F_{ij}(t) \rangle = \int_{\Omega} P_{ZZ}^L Q_{ij}^L d\Omega w(t), \quad (\text{C } 1)$$

where the polarization of the exciting light is parallel to the laboratory Z -axis.

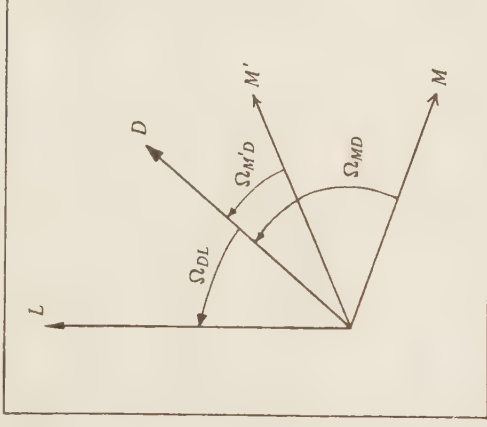


Fig. 20 Schematic picture of the laboratory (L), director (D) and molecular fixed frame. The orientation of the molecular frame at times of excitation and emission is denoted M and M' , respectively.

The transformations from the molecular frame (M) to the director of the aggregate (D) and further to the laboratory frame (L) are illustrated in Fig. 20.

The isotropic average with respect to Ω_{DL} of

$$\langle F_{ij}(t) \rangle = \int_{\Omega_{DL}} \frac{1}{8\pi^2} \left(\frac{1}{3} \text{Tr} \tilde{P} + \frac{2}{3} \sum_n P_n^D D_{0n}^{(2)*}(\Omega_{DL}) \right) Q_{ij}^L(\Omega_{DL}) d\Omega_{DL} w(t) \quad (\text{C } 2)$$

yields

$$\langle F_{ij}(t) \rangle = \begin{pmatrix} \text{Tr} \tilde{P} \text{Tr} \tilde{Q} - \frac{2}{3} \sum_n P_n^D Q_{-n}^D(-1)^n & 0 & 0 \\ 0 & \text{Tr} \tilde{P} \text{Tr} \tilde{Q} - \frac{2}{3} \sum_n P_n^D Q_{-n}^D(-1)^n & 0 \\ 0 & 0 & \text{Tr} \tilde{P} \text{Tr} \tilde{Q} + \frac{2}{3} \sum_n P_n^D Q_{-n}^D(-1)^n \end{pmatrix} \frac{w(t)}{9} \quad (\text{C } 3)$$

Further

$$\begin{aligned} & \langle \sum_n P_n^D Q_{-n}^D (-1)^n \rangle \\ &= \int_{\Omega_{MD}} \sum_{n,m} (-1)^n P_m^M Q_0^M D_{nm}^{(2)*}(\Omega_{MD}) D_{-n0}^{(2)*}(\Omega_{MD}) f(\Omega_{MD}) \\ & \quad \times g(\Omega_{MD}) |\Omega_{MD'}| t) d\Omega_{MD} d\Omega_{MD'}, \end{aligned} \quad (C4)$$

if the molecular z -axis is parallel to the emission dipole moment.

At $t = 0$ equations (C4) and (5.9) give

$$\int_{\Omega_{MD}} \sum_{n,m} (-1)^n P_m^M Q_0^M D_{nm}^{(2)*}(\Omega_{MD}) D_{-n0}^{(2)*}(\Omega_{MD}) f(\Omega_{MD}) d\Omega_{MD}, \quad (C5)$$

which after the substitution $D_{-n0}^{(2)*}(\Omega_{MD}) = (-1)^{-n} D_{n0}^{(2)}(\Omega_{MD})$ and summation over n becomes

$$\int_{\Omega_{MD}} P_0^M Q_0^M f(\Omega_{MD}) d\Omega_{MD} = p^2 q^2 D_{00}^{(2)}(\delta). \quad (C6)$$

δ is the angle between the absorption and emission dipole moments (cf. equation 5.7).

At $t = t_\infty$ equations C4 and 5.10 give

$$\begin{aligned} \langle \sum_n P_n^D Q_{-n}^D (-1)^n \rangle &= \int_{\Omega_{MD}} \sum_{n,m} (-1)^n P_m^M D_{nm}^{(2)*}(\Omega_{MD}) d\Omega_{MD} \\ & \quad \times \int_{\Omega_{MD'}} Q_0^M D_{-n0}^{(2)*}(\Omega_{MD'}) f(\Omega_{MD'}) d\Omega_{MD'}. \end{aligned} \quad (C7)$$

If f is uniaxial

$$\langle \sum_n P_n^D Q_{-n}^D (-1)^n \rangle = \sum_m P_m^M \langle D_{0m}^{(2)*}(\Omega_{MD}) \rangle Q_0^M \langle D_{00}^{(2)}(\Omega_{MD}) \rangle. \quad (C8)$$

A combination of equations C3, C8 and the fluorescence anisotropy

$$r = \frac{F_{ZZ} - F_{XX}}{F_{ZZ} + 2F_{XX}}$$

yields

$$r(0) = \frac{2}{3} D_{00}^{(2)}(\delta), \quad (C9)$$

$$r(t_\infty) = \frac{\sum_m P_m^M \langle D_{0m}^{(2)*}(\Omega_{MD}) \rangle \langle D_{00}^{(2)}(\Omega_{MD}) \rangle}{\text{Tr } \tilde{P}}. \quad (C10)$$

Thus generally $r(t_\infty)$ is not a simple function of S but contains a product of different order parameters.

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II

Linear Dichroism as a Tool for Studying Molecular Orientation in Membrane Systems.

2. Order Parameters of Guest Molecules from Linear Dichroism and Nuclear Magnetic Resonance

Lennart B.-Å Johansson,*† Åke Davidsson,† Göran Lindblom,† and Bengt Nordén‡

Department of Inorganic Chemistry 1 and Department of Physical Chemistry 2, University of Lund, Chemical Center, S-220 07 Lund, Sweden (Received January 10, 1978; Revised Manuscript Received May 25, 1978)

A linear dichroism (LD) method using macroscopically aligned samples was recently developed for studying biological model membranes. We show here that for these systems the influence from multiple reflections must be considered when chromophore orientation is low. A comparison between order parameters obtained from LD and NMR has also been performed, by studying the ^2H NMR quadrupole splittings of perdeuterated chromophores. The benzene plane was found to be preferentially oriented parallel to the amphiphilic surfaces of the lamellar phase of cetyltrimethylammonium bromide (CTAB)/1-hexanol/water and of the hexagonal phase of CTAB/water. In the lamellar liquid crystalline phase composed of monooctanoin/water, the molecular plane of benzene and naphthalene tended to be oriented perpendicular to the lamellar surface. The order parameters from LD were in satisfactory agreement with those obtained from NMR. Studies of benzene in aqueous micellar solutions of CTAB in Couette flow indicated that the rod-shaped micelles were inefficiently aligned. Lecithin liposomes oriented by flow showed distorted absorption spectra.

Introduction

Linear dichroism (LD) spectroscopy is a useful method to study orientation of chromophoric molecules in different macroscopically alignable systems.¹⁻⁵ The applicability of this method for investigations of natural¹ and model² membranes is therefore very promising. Recently we demonstrated in a systematic LD study² that the molecular orientation can be determined for a chromophore in lyotropic lamellar liquid crystalline phases, where the lamellar system was aligned between quartz plates. The LD was measured at a nonperpendicular angle between the plane of the plates and the incident light beam. When the chromophore orientation in the lamellae is relatively large its order parameter, describing an average orientation, can be determined straightforwardly with the method described in ref 2. However, if the molecular order is small (having an order parameter of less than about 0.05) it can be shown that the effect of reflections at the air-quartz and quartz-sample boundaries has to be considered. Here this orientation independent effect is quantitatively taken into account so that chromophores with low orientation in various membranes also can be studied.

Several membrane lipids, as, for example, lecithin, dispersed in water form large asymmetric multilamellar aggregates (so-called liposomes⁶). Previously we have shown³ that long rodlike micelles of cetyltrimethylammonium bromide (CTAB) in water can be aligned by flow in a Couette cell permitting an examination of the

orientation of a solubilized chromophore in the micelles. Encouraged by these results we found it convenient to use this flow method for alignment of liposomes to study chromophore orientation in model membranes. However, as will be briefly discussed here, the interpretation in terms of molecular orientation of such LD results is subjected to formidable difficulties. Molecular orientation can also be studied by NMR⁷ using for instance deuterium labeled compounds. Since no macroscopical alignment of the sample is needed in this method it has been used for an independent determination of the molecular orientations which are then compared with the results obtained from LD.

In this work several amphiphilic systems have been studied: micellar and liposome solutions and hexagonal and lamellar liquid crystalline phases. The deuterated chromophoric molecules solubilized in the different systems have been benzene and/or naphthalene.

Experimental Section

Linear dichroism was measured with a modified Jasco J-40 circular dichroism spectrometer on aligned lamellar mesophase samples² and on micellar solutions and hexagonal mesophase samples in a flow apparatus³ (Couette cell), see Figure 1, as described previously. The lamellar samples were aligned between quartz plates and the samples were then studied at an angle of inclination, $\omega = \pi/4$, between the propagation vector of the light and the plane of the quartz plates.² The temperature was kept at 28 °C by using a thermostated copper block.

Birefringence was determined by observing the apparent decrease in circular dichroism of an optically active sample

* Department of Physical Chemistry 2.

† Department of Inorganic Chemistry 1.

‡ Department of Physical Chemistry 2.

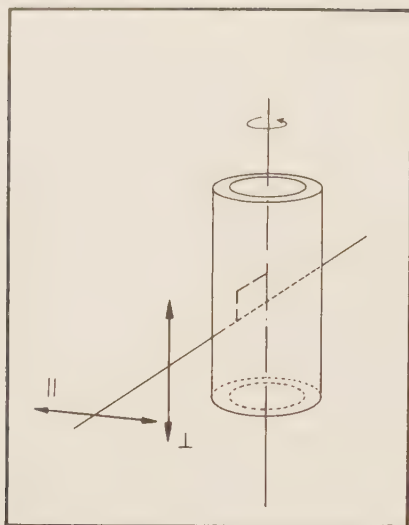


Figure 1. Geometry of measuring arrangement for the Couette cell.

in a circular dichroism spectrometer when the oriented sample was introduced in front of it in the light path. The phase difference, ψ , between ordinary and extraordinary rays resulting from the passage through the birefringent sample is given by $\psi = (2\pi/\lambda) d|\Delta n_\omega|$; Δn_ω is the effective birefringence at the angle ω , d is the optical pathlength, and λ is the wavelength of light. Δn_ω is directly obtained from

$$|\Delta n|_\omega = \frac{\lambda}{4d} |(CD_0 - CD)/CD_0| \quad (1)$$

where CD_0 is the circular dichroism in the absence of the birefringent specimen.

Refractive indices of the quartz and the lamellae were determined according to the methods developed in ref 8.

The ^2H NMR studies were made with a Bruker 322 s pulsed NMR spectrometer operating at 13.8 MHz in the Fourier transform mode. The NMR spectra were obtained according to the method developed by Davis et al.⁹ (see Figure 2). In most cases about 100 000 transients were accumulated. The dwell time was 20 μs .

Cetyltrimethylammonium bromide was purchased from Merck (p.a. quality) and was used without purification. The perdeuterated benzene and naphthalene were bought from BDH and Aldrich, respectively.

Results and Discussion

Linear Dichroism of Macroscopically Aligned Lamellar Samples. Lamellar phases containing chromophoric molecules can be efficiently aligned as thin layers between quartz plates.² The director of the lamellar system is then perpendicular to the plates. This is a prerequisite for any determination of the molecular orientation with respect to the lamellae. Figure 3 is a schematic picture of a bilayer inclined with respect to the direction of optical propagation.

The orientations of the electric field vectors of the incident light, i.e., the laboratory arrangement, appear from the parallel and perpendicular notations in Figure 3. The order parameter, S , is related to the measurable quantities, the absorbance of a random sample, A_r , and the linear dichroism, $LD = A_{||} - A_{\perp}$, through the equation²

$$(LD/A_r)_\omega = 3Sn_2^{-2} \cos^2 \omega (1 - n_2^{-2} \cos^2 \omega)^{-1/2} \quad (2)$$

Here n_2 is the isotropic refractive index of the lamellar phase and $S = 1/2(3 \cos^2 \theta_{DM} - 1)$ where θ_{DM} is the angle between the director (i.e., the normal to the bilayer) and

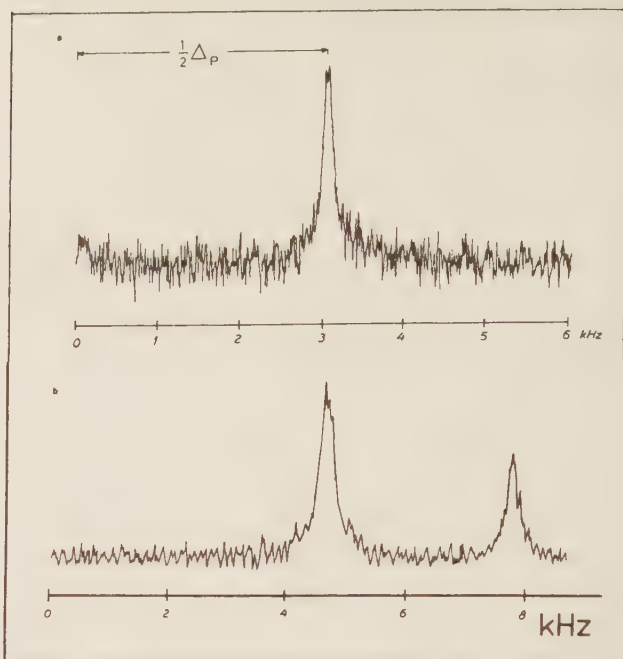


Figure 2. ^2H NMR quadrupole splittings of (a) C_6D_6 in a 20% aqueous CTAB solution and (b) C_{10}D_8 in a lamellar liquid crystalline phase composed of 60% monooctanoin and 40% water. Only half of the splittings are shown since the spectrum is symmetric around the resonance frequency (13.8 MHz, temperature 28 $^\circ\text{C}$).

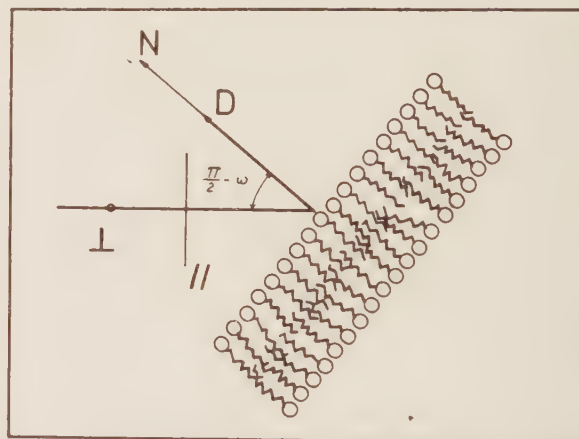


Figure 3. Schematic picture showing the polarizations and the direction of propagation of the incident light with respect to the bilayer normal. The normal, N , to the plate coincides with the director, D , of the lamellar system.

the absorbing transition dipole moment. The absorbance, A , measured at normal incidence to the quartz plates, is related² to the absorbance A_r through $A_r = A + LD_{\omega=0}/3$.

With the intention of making the optical analysis more complete we have examined the influence of birefringence and of polarized multiple reflections on the measured LD for lamellar systems.

Let us first discuss the effect of birefringence. We denote the refractive index to be observed when the electric vector of light is parallel to the director, n_D , and $n_{\perp}$ when the electric vector is perpendicular to the director. The birefringence is defined as $\Delta n = n_{\perp} - n_D$. The refractive index $n_{\omega'}$ is given by

$$n_{\omega'}^{-2} = n_D^{-2} \cos^2 \omega' + n_{\perp}^{-2} \sin^2 \omega' \quad (3)$$

where $(\pi/2 - \omega')$ is the angle between the refracted rays and the normal to the quartz plates. Consequently the refractive indices in the perpendicular and parallel di-

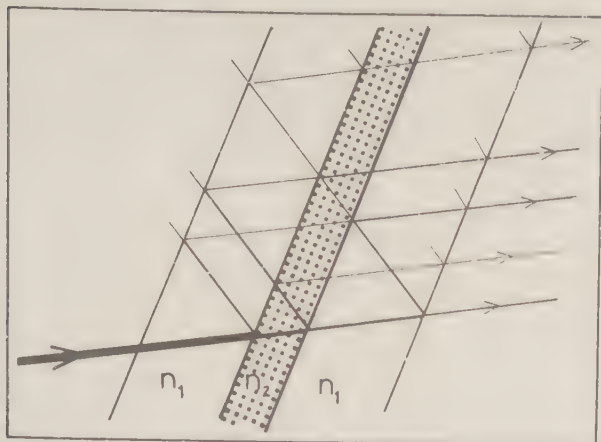


Figure 4. Scheme showing the different reflections taken into account by eq 8.

reflections are different leading to that the optical paths through the lamellae of the perpendicular and parallel polarized rays, will be slightly different. Equation 2 is therefore corrected with respect to a small birefringence by McLaurin expansion to the first order in Δn :

$$\begin{aligned} (LD/A_r)_\omega = & 3Sn_\perp^{-2}(1 - n_\perp^{-2} \cos^2 \omega)^{-1/2} \cos^2 \omega + \\ & 2\Delta nn_\perp^{-3}(1 - n_\perp^{-2} \cos^2 \omega)^{-1/2} [\frac{1}{2}(1 - \\ & n_\perp^{-2} \cos^2 \omega)^{-1}(S(3n_\perp^{-2} \cos^2 \omega - 1) + 1) + \\ & 3S] \cos^2 \omega' \cos^2 \omega \quad (4) \end{aligned}$$

By using eq 4 we can now estimate the effect of the birefringence on the observed LD. Let us therefore choose some experimental possible values for the parameters involved. For instance, with $\Delta N = -0.01$, $n_\perp = 1.5$, $\omega = 45^\circ$, and $S = 0.05$ the second term of this expression is less than 3% of the first term (since $45^\circ < \omega' < 90^\circ$) which is usually a negligible effect. However, in cases of low S and strong birefringence the effect is important. Notice that for $\Delta n < 0$ and $S > 0$ the measured LD/A_r is smaller than predicted by eq 2, whereas for $\Delta n > 0$ and $S > 0$ it is larger.

The second optical effect which has to be considered here is polarized multiple reflections at the four boundaries of the specimen (see Figure 4). This multiple reflection affects the LD signal and is due to the difference in Fresnel reflections for the two polarization modes (parallel and perpendicular) at these boundaries. Since the transmitted light intensities are different for the different modes we shall expect to observe an LD also for an absorbing random sample between quartz plates. The intensity of a reflected beam from a boundary between two media with refractive indices n_1 and n_2 is given by the Fresnel equations^{10,11}

$$\begin{aligned} R_\perp &= \left(\frac{n_1 \cos \phi_1 - n_2 \cos \phi_2}{n_1 \cos \phi_1 + n_2 \cos \phi_2} \right)^2 \\ R_\parallel &= \left(\frac{n_2 \cos \phi_1 - n_1 \cos \phi_2}{n_2 \cos \phi_1 + n_1 \cos \phi_2} \right)^2 \quad (5) \end{aligned}$$

ϕ_1 and ϕ_2 are the angles between the incident beam and the refracted beam, respectively, and the normal to the boundary surface. Only the real part of the complex refractive index is considered (the imaginary part is usually of the order of 10^{-5} – 10^{-6}).²

It is possible to show that there is a rapid convergence with increasing number of reflections, and in most practical cases it should be sufficient to consider only the reflections shown in Figure 4, i.e. four reflections. The total transmitted intensity, I_ν , is then given by

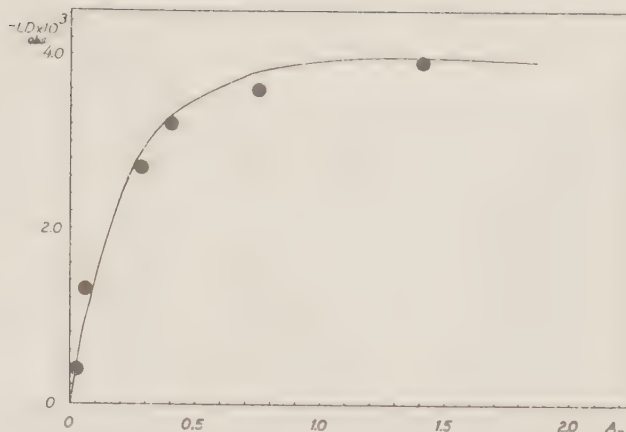


Figure 5. Multireflection LD vs. the sample absorption A_r (254-nm band of random solution of benzene in ethanol between quartz plates; thickness 1 mm, tilt angle 45°): (—) calculated from eq 8 (see text); (●) obtained from experiment.

$$I_\nu/I_{0\nu} = (1 - R_{1\nu})^2(1 - R_{2\nu})^2 10^{-A_\nu} [1 + 2R_{1\nu}R_{2\nu} + 10^{-2A_\nu} \{R_{2\nu} + R_{1\nu}(1 - R_{2\nu})^2\}^2] \quad \nu = \parallel, \perp \quad (6)$$

The indices denote the reflections at the air-quartz (1) and quartz-sample (2) boundaries. The observed LD (LD_{obsd}) is thus composed of the LD due to the absorbing chromophore ($A_\parallel - A_\perp$) and a contribution (m) from the polarized reflections:

$$LD_{\text{obsd}} = \log(I_{0\parallel}/I_\parallel) - \log(I_{0\perp}/I_\perp) - \{\log(I_{0\parallel}/I_\parallel)_{A=0} - \log(I_{0\perp}/I_\perp)_{A=0}\} = LD + m \quad (7)$$

LD_{obsd} is obtained as the peak height above a baseline extrapolated from the transparent regions surrounding the absorption band. The second term in the bracket ($\{\}$) is the reflection linear dichroism obtained for a nonabsorbing sample.

Combining eq 6 and 7 we obtain

$$m = \frac{1}{\ln 10} \{ [R_{2\perp} + R_{1\perp}(1 - R_{2\perp})^2](10^{-2A_\perp} - 1) - [R_{2\parallel} + R_{1\parallel}(1 - R_{2\parallel})^2](10^{-2A_\parallel} - 1) \} \quad R_{j\nu}R_{i\nu} \ll 1 \quad (8)$$

This equation can be used to estimate the order of the effect of a possible multiple reflection on the LD_{obsd} signal by setting $A_\parallel = A_\perp$.

Figure 5 shows LD_{obsd} vs. A_r observed for an isotropic benzene-ethanol solution between quartz plates together with calculated $LD_{\text{obsd}} = m$ values from eq 8. As can be inferred from this figure the agreement between the observed and the calculated data is convincing. The non-linear characteristics of the reflection LD makes it easy to discriminate this effect from ordinary orientation LD (for instance by the observation of a flattening of the absorption peaks). The main objective of most LD studies of a model membrane system is to determine the orientation of the chromophore, i.e., to determine the order parameter, S . Unfortunately, S cannot be calculated directly from the equations obtained above, since m in eq 8 is dependent on the chromophore orientation through $A_\parallel$ and $A_\perp$. However, in spite of this inconvenience, S can be obtained by an iteration procedure from eq 5, 7, and 8. Figure 6 illustrates the relative effect of the multiple reflection on LD as a function of the order parameter. As can be seen from this figure multiple reflection starts to be a serious problem for small S values.

The orientation of benzene was studied in two different lamellar mesophase systems, one composed of cetyltri-

TABLE I: Orientation of Benzene and Naphthalene in Different Amphiphilic Systems from NMR and LD

amphiphilic system compn ^a	phase ^b	molecule studied (mmol L ⁻¹)	LD		² H NMR S ^c	molecular axis ^d
			orientation techn	S ^c		
C ₈ -1-monoglyceride (60)	D	C ₆ D ₆ (500)	aligned lamellae between quartz plates	-0.09	0.10	C ₅
H ₂ O (40)						
C ₈ -1-monoglyceride (60)	D	C ₁₀ D ₈ (500)	aligned lamellae between quartz plates	0.11	0.12	long
H ₂ O (40)				0.06	0.07	short
C ₁₆ -N(CH ₃) ₃ Br (20)	L ₁	C ₆ D ₆ (20-100)	Couette flow	0.024	0.16	C ₆
H ₂ O (80)						
C ₁₆ -N(CH ₃) ₃ Br (30)	E	C ₆ D ₆ (20-100)	Couette flow	0.025	0.16	C ₆
H ₂ O (70)						
C ₁₆ -N(CH ₃) ₃ Br (35)	D	C ₆ D ₆ (500)	aligned lamellae between quartz plates	0.07	0.10	C ₆
1-hexanol (15)						
H ₂ O (50)						

^a Weight percent given in parentheses. Temperature 28 °C. ^b Phase notations according to Ekwall: lamellar (D), hexagonal (E), and micellar (L₁).¹² ^c The order parameter *S* refers to the indicated molecular axis and the local axis perpendicular to the aggregate surface. ^d Axis to which *S* value refers.

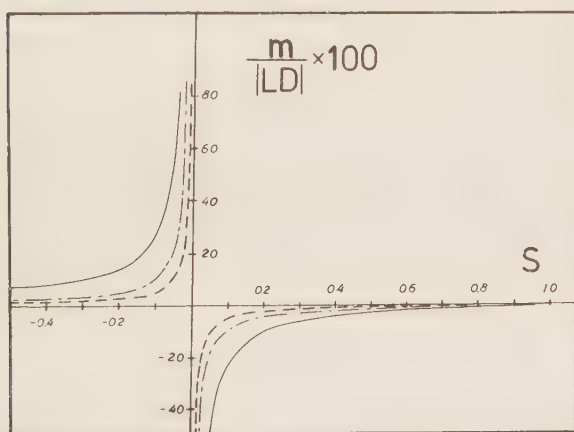


Figure 6. The relative effect of multiple reflection (*m*) on the linear dichroism (*LD*) according to eq 8: *A_r* = 1.0 (—), 0.5 (---), and 0.05 (-.-); *n*₁ = 1.52 (quartz at 250 nm), *n*₂ = 1.50.

methyammonium bromide, hexanol, and water and the other one was composed of monooctanoin and water. Concerning the phase diagrams for these see for example the review by Ekwall.¹² For the monooctanoin system naphthalene also was solubilized and studied by LD. The experimental data obtained are summarized in Table I. For benzene (*D*_{6h} symmetry) the LD and *A_r* were observed at 250–260 nm, and this band is degenerate and in-plane polarized. For naphthalene (*D*_{2h} symmetry) the bands at 250 and 290 nm are used to calculate the order parameters of the long and short axis. The 290-nm band contains a known contribution from both long and short axis polarizations and the 250-nm absorption has long axis polarization.¹³ This purity of the 250-nm band has been tested by investigating the pure long axis polarized transition at 220 nm. LD/*A_r* obtained at 220 nm was 0.31 ± 0.02, which is in good agreement with the value obtained at 250 nm (0.30 ± 0.01). The order parameter for both chromophores were then obtained from the equations given above (Table I). From Table I it can be seen that the order parameter obtained for benzene is rather small and a correction for multiple reflection was performed. It can also be inferred from this table that the *S* values for both CTAB and the monoglyceride systems are of the same order of magnitude but that the sign is different. These data indicate that the plane of the benzene molecule tends to orient parallel to the lamellar surface for the CTAB system but perpendicular to the surface for the monooctanoin system. Similarly, it can be concluded that naphthalene tends to be oriented perpendicular to the lamellar surface. Note also that the order parameter is

positive for the short-axis as well as for the long-axis polarized transitions.

Linear Dichroism in Couette Flow. The Couette flow technique with alignable elongated aggregates has been demonstrated previously.^{1,3-5} Solutions containing rodlike aggregates such as, e.g., micelles³ or liposomes can be aligned by shear forces in the gap between the cylinders in the arrangement shown in Figure 1. If the rods are perfectly aligned by the flow with their long axes parallel to the direction of flow, the order parameter, *S*, of the chromophore molecule (axis of the absorbing transition dipole) with respect to the long axis of the rod is given by the equation²

$$S = (LD/A_r)/3 \quad (9)$$

where *A_r* again is the absorbance of the random sample.

In a previous investigation³ of the orientation of benzene in long rodlike CTAB micelles we found that the LD above a certain shear gradient was independent of the gradient. It was also observed that if the CTAB (benzene) aqueous solution was subjected to prolonged shear the LD signal did not relax immediately back to zero (random orientation) but stayed constant for several hours (ca. 10 h). Almost the same orientation is also obtained from Couette flow LD for benzene solubilized in a hexagonal¹² CTAB liquid crystalline phase, consisting of long cylindrical micelles in a hexagonal array. Taking these experimental data together led us to the conclusion that the rodlike micelles were perfectly aligned along the flow direction. Thus the order parameters calculated, which are summarized in Table I, should describe the average orientation of the benzene molecule relative to the long axis of the rod. (Unfortunately, there is an error in ref 3 giving an order parameter a factor of 100 too low.) However, as is shown below, NMR data on benzene orientation in these systems obtained by us (Table I) and others¹⁴ indicate that perfect orientation along the flow direction is not obtained in these experiments. We cannot give an unambiguous explanation for this behavior at this stage of the investigation.

As mentioned in the Introduction, we thought that a lecithin liposom dispersion might be a good model membrane system, useful for convenient Couette LD measurements of chromophore orientation. This proved to be an optimistic expectation, because here new serious difficulties appeared which probably have to be considered also in other similar systems studied by other workers.^{15,16} Thus it was found for liposomes that the absorption spectrum was severely distorted for different chromophoric molecules studied, solubilized in the bilayer of the liposomes. The effect observed has been explained previously¹⁷ and it is caused by a statistical absorption of

the large aggregates in the two phase system (liposomes + water). Therefore we must conclude from these experimental findings that if one would like to obtain information about the orientation on the molecular level, i.e., orientation in the model membrane system, the Couette flow technique cannot be used straightforwardly.

Deuteron Nuclear Magnetic Resonance Studies. NMR provides an independent way of determining the average orientation of a solubilized molecule. In contrast to the case with LD, we can here only obtain the absolute value of the order parameter. It is usually also difficult to reach the very low concentration ranges which are accessible with the LD technique for strongly absorbing chromophores. An advantage with NMR, however, is that unoriented samples can be used. The ^2H nucleus, having a spin quantum number equal to 1 and a quadrupole moment, gives rise to two peaks in the NMR spectrum, provided the environment of the nucleus is anisotropic.¹⁸ The distance measured between these two peaks is called a quadrupole splitting, and from this an order parameter can be calculated (see Figure 2, where the splittings are shown for benzene and naphthalene). In order to be able to compare order parameters obtained from LD and NMR measurements, they must be related to the same axes in the molecule. The NMR experiment gives an order parameter ($S(\text{C-D})$), for the average order of the C-D bond relative to the director. The LD order parameter describes the corresponding average orientation of the transition dipole moment which is directed along a symmetry axis in the plane of the molecule. Therefore a transformation of the axis to which the order parameter refers is necessary for naphthalene, but not for benzene where the transition dipole and the C-D bond are parallel to axes in the same molecular coordinate system. In benzene it is convenient to present the result in terms of the order parameter of the sixfold symmetry axis which is $S(\text{C}_6) = -2S(\text{in-plane transition}) = -2S(\text{C-D})$. For naphthalene the transformation is summarized below.

The NMR quadrupole splitting can be written⁷

$$\Delta_\nu = \frac{3}{4}\nu_Q|S(\text{C-D})| \quad (10)$$

where $S(\text{C-D})$ describes the average orientation of the C-D bond relative to the director.¹⁹

$$S(\text{C-D}) = \overline{D_{00}^{(2)}(\Omega_{\text{DM}})} = \frac{1}{2}(3 \cos^2 \theta_{\text{DM}} - 1) \quad (11)$$

where $D_{00}^{(2)}$ is a Wigner rotation matrix element and Ω_{DM} are the Euler angles specifying the transformation from the molecular frame (M) to the director frame (D). For naphthalene the M frame (the C-D bond) can be transformed to a new molecular frame M' , where the z axis is along the long axis by²⁰

$$\begin{aligned} \overline{D_{00}^{(2)}(\Omega_{\text{DM}})} &= \sum_q \overline{D_{0q}^{(2)}(\Omega_{\text{DM}'})} D_{q0}^{(2)}(\Omega_{\text{M}'\text{M}}) = \\ &= \overline{D_{00}^{(2)}(\Omega_{\text{DM}'})} D_{00}^{(2)}(\Omega_{\text{M}'\text{M}}) + 2\overline{D_{02}^{(2)}(\Omega_{\text{DM}'})} D_{20}^{(2)}(\Omega_{\text{M}'\text{M}}) = \\ &= \frac{1}{2}(3 \cos^2 \beta - 1)\overline{D_{00}^{(2)}(\Omega_{\text{DM}'})} + 2[(3/8)^{1/2} \sin^2 \beta \overline{D_{02}^{(2)}(\Omega_{\text{DM}'})}] \end{aligned} \quad (12)$$

The quadrupole splittings obtained for naphthalene- d_8 can then be used to calculate the new order parameter $\overline{D_{00}^{(2)}(\Omega_{\text{DM}'})}$ for the molecular long axis. From the measured quadrupole splittings, order parameters were calculated by means of eq 10 (the quadrupole coupling constant was set equal to 197 kHz²¹). Since only the absolute value of the order parameter can be obtained by NMR the sign may be positive or negative, and $S(\text{C-D})$ is equal to ± 0.065 and ± 0.106 for naphthalene, and ± 0.052 for benzene solubilized in a monoglyceride lamellar mesophase (cf. Table I). Thus from eq 12 the following eight order parameters for the long axis of naphthalene were obtained: ± 0.12 ,

± 0.05 , ± 0.45 , and ± 0.12 . A similar calculation was done for the order parameter of the in-plane short axis of naphthalene.

Comparison between the LD and NMR Results. Order parameters for benzene solubilized in micelles and in hexagonal and lamellar mesophases have been obtained from LD and ^2H NMR experiments. Naphthalene was studied with both methods only for a lamellar liquid crystalline sample. All the results are summarized in Table I. Note that LD but not NMR gives the order parameter with its sign. It can be inferred from Table I that for benzene in both the lamellar phases studied NMR and LD data are in very good agreement. It was shown in the previous section that, for naphthalene, NMR gives a set of several values for the order parameter. From an inspection of such S values in the sets for the long and short axes of naphthalene it is found that one value, respectively, is very close to the appropriate order parameter determined with LD (see Table I). It can therefore be concluded, since the NMR and LD S values are almost the same for the lamellar systems studied that the samples are very well aligned between the quartz plates. This conclusion is also supported by the fact that the samples always had an LD equal to zero when viewed at normal incidence. Furthermore it was found that the polarization microscope immediately revealed if the samples contained unaligned domains. A high macroscopic lamellar orientation was also indicated by the very sharp angular dependence of the proton NMR spin echo amplitude observed on a stack of lamellae between glass plates.²² The discrepancy observed for the Couette LD and NMR S values is surprising since investigations of LD on the shear gradient³ showed that LD above a threshold value was independent of the gradient. This result suggests a complete orientation of the micellar aggregates in the Couette flow. However, the approximately fivefold lower order parameter obtained with LD compared to NMR (see Table I) can only be explained by a deviation from a perfect alignment of the long rodlike micelles in the flow direction.

Concluding Remarks

The satisfactory agreement between the order parameters obtained here from LD and ^2H NMR quadrupole splittings for model membrane systems suggests that LD can be used for determination of the order parameter even if the molecular orientation is low. A necessary condition to obtain this average molecular orientation of the chromophore is an almost complete macroscopic alignment of the lamellar system.

For the Couette flow technique two problems arise which are not easily circumvented, namely, the amphiphilic aggregates are not perfectly oriented and for large aggregates such as liposomes (two phase system) absorption statistics occurs. Unfortunately, for the kind of systems discussed here we have not been able to find corrections for any of these effects.

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III

Electronic Transitions in the Isoalloxazine Ring and Orientation of Flavins in Model Membranes Studied by Polarized Light Spectroscopy[†]

Lennart B.-Å. Johansson, Åke Davidsson,* Göran Lindblom, and K. Razi Naqvi

ABSTRACT: The orientation of flavin mononucleotide (FMN) in model membranes and the directions of the transition moments of the first three bands in the electronic absorption spectrum of the oxidized form of the isoalloxazine ring have been determined by means of linear dichroism and polarized

fluorescence spectroscopy. Measured counterclockwise relative to the axis connecting the two nitrogens in the central ring (considered positive when going in the direction from $-\text{CN} <$ to $\geq \text{N}$), these angles are $58 \pm 4^\circ$ (450-nm band), $97 \pm 3^\circ$ (350-nm band), and $119 \pm 2^\circ$ (260-nm band).

Biological membranes, being well organized structures, owe many, if not all, of their functions to the spatial arrangement of their constituents. Information about molecular orientation is therefore necessary for unravelling the functional mechanisms of a membrane. This pertains in particular to the enzymes (flavoproteins and cytochromes) in the respiratory chain. Here we have studied the flavin mononucleotide (FMN), which is a prosthetic group of many flavoproteins.

A technique that has long been used for studying the orientation of chromophores in biological membranes is linear dichroism (LD) in which one studies the absorption of plane-polarized light by an ensemble of oriented chromophores (Schmidt, 1938; Johansson et al., 1978). Given the knowledge of the orientation, the directions of the transition moments of the different electronic transitions giving rise to various absorption bands can be determined; conversely, if the directions of the transition moments are already known, information regarding the orientation of the chromophore can be extracted.

For highly symmetric molecules, the directions of the transition moments are relatively easy to ascertain; unfortunately, flavins, like most other biological chromophores, do not fall in this convenient category, which makes the determination of the directions of the transition moments a demanding task, but a task that must nonetheless be undertaken if the orientation of FMN is to be studied.

In the absorption spectrum of an oxidized flavin, the first three bands, to be denoted here as I, II, and III, occur at 450, 375–330 (depending on the solvent polarity), and 270 nm, respectively; all three have been assigned to $\pi^* \leftarrow \pi$ transitions (Weber, 1966; Palmer & Massey, 1968; Sun et al., 1972; Yu et al., 1976). Though a great deal of attention has been paid to the spectroscopic aspects of the flavins, no previous work has grappled with the problem which is considered here, viz., the location, in the molecular framework, of all three transition moments. To reach this goal, we have studied the fluorescence polarization of lumiflavin, riboflavin, and FMN and the electric linear dichroism (ELD) of lumiflavin. The results of this study have allowed us to grasp, and describe, the orientation of FMN in model membranes.

Materials and Methods

The absorption spectra were recorded on a Cary 118C spectrometer. Measurements of LD and ELD (Davidsson &

[†] From the Division of Physical Chemistry 2, Chemical Centre, University of Lund, S-220 07 Lund 7, Sweden (L.B.-Å.J. and G.L.), Inorganic Chemistry 1, Chemical Centre, University of Lund, S-220 07 Lund 7, Sweden (Å.D.), and the Department of Physics, NLHT, University of Trondheim, N-7000 Trondheim, Norway (K.R.N.). Received April 6, 1979. This work was supported by the Swedish Natural Science Research Council and the Norwegian Research Council for Science and Humanities.

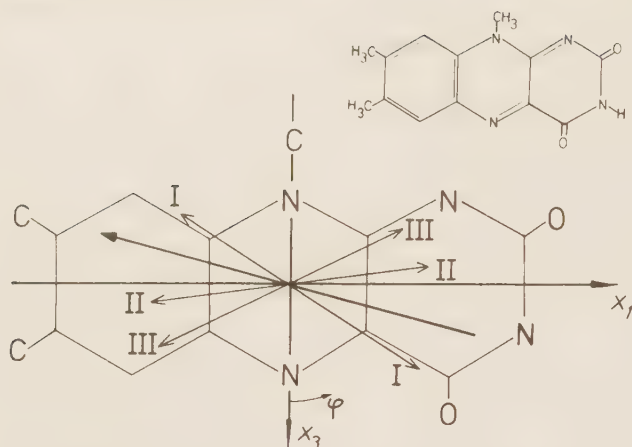


FIGURE 1: Skeleton of the isoxaloxazine ring with the $x_1x_2x_3$ coordinate system. The transition moments I, II, and III and the angle ϕ are shown. The molecular structure of lumiflavin is shown in the upper right of the figure.

Norden, 1977; Davidsson, 1978) were performed with a modified Jasco J-40 CD spectrometer. Polarized fluorescence spectra were recorded on a purpose-built instrument of standard design; the band-pass of the excitation monochromator and the analyzing monochromator was limited to 10 nm. The degree of polarization, p , of the fluorescence (monitored at 520 nm) was determined in the usual manner (Albrecht, 1961), and the measured values of p were converted to "intrinsic" values, denoted by p_i , by introducing a randomization factor in the manner described by Albrecht (1961).

Lumiflavin and flavin mononucleotide (FMN; 7,8,10-trimethylisoxaloxazine) were purchased from Sigma, and riboflavin was from Koch-Light. Monooctanoin was synthesized at "Syntestjänst", Chemical Center, Lund.

The refractive indexes at 23 °C (the temperature at which all spectra were recorded) of the monooctanoin samples (containing 70% monooctanoin and 30% water) were 1.43 at 589 nm and 1.50 at 250 nm. The solvent used in the ELD measurements was a 1:1 mixture by volume of CCl_4 and CHCl_3 . The dielectric constant of this was measured with a WTW dipolmeter DMOI to be 3.30 ± 0.05 .

Results

Fluorescence Measurements. In the following, we will be interested in the polarization excitation spectrum, i.e., in the variation in p_i with λ_e , the wavelength of excitation. No significant difference was found in the polarization spectra of lumiflavin, riboflavin, and FMN; for our purpose, therefore, the three molecules may be adjudged to be spectroscopically akin, all three faithfully representing the spectral properties of the parent molecule, the oxidized form of the isoxaloxazine ring. The results of our study, which are in excellent agreement with the data reported by Chen & Bowman (1965) and by Gordon-Walker et al. (1970) can be briefly stated: $p_i(450) = 0.5$, $p_i(375) = 0.345 \pm 0.01$, and $p_i(260) = 0.100 \pm 0.04$, where λ_e is indicated in the parentheses in nanometers.

The angle, θ , between the absorption dipole and the emission dipole, calculated by using the relation shown in eq 1 and

$$p_i = (3 \cos^2 \theta - 1) / (3 + \cos^2 \theta) \quad (1)$$

inserting the above figures for $p_i(375)$ and $p_i(260)$, comes out to be $29 \pm 1^\circ$ and $48 \pm 3^\circ$, respectively. The quantities we seek are $\theta_{I,II}$ and $\theta_{I,III}$, where the former is the angle between the transition moments for bands I and II and the latter is the corresponding angle for bands I and III; $\theta_{I,II}$ and $\theta_{I,III}$ can be equated to 29 and 48° , respectively, only if we assume that,

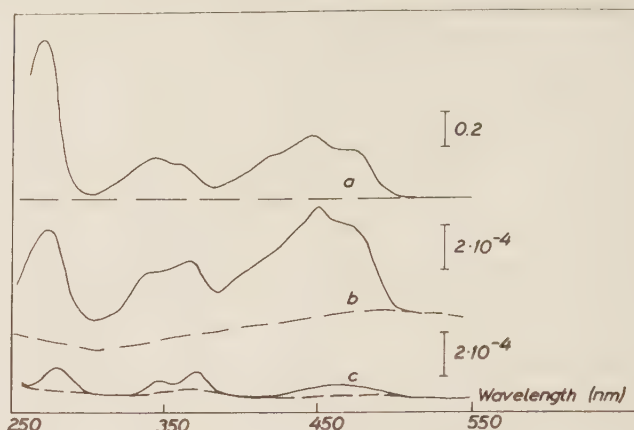


FIGURE 2: (a) Absorption spectrum of lumiflavin in a mixture of CHCl_3 - CCl_4 . (b) ELD for the case when the linear polarized light is parallel to the applied electric field. $E_{\text{app}} \approx 10^6$ V/m. (c) Same as in part b, but at the angle 54.7° between the polarized light and the applied field.

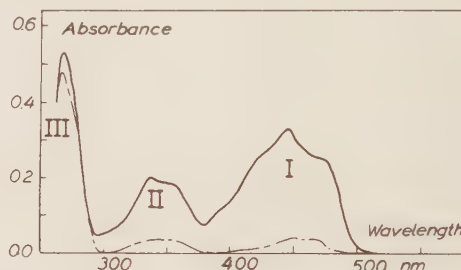


FIGURE 3: Absorbance parallel (—) ($A_{\parallel\mu}$) and perpendicular (---) ($A_{\perp\mu}$) to the electric dipole moment.

at 375 and 260 nm, absorption arises solely from one transition. A simple calculation shows that even if 1% (1.5%) of the total absorption at 375 nm can be traced to the tail of band I, $\theta_{I,II}$ increases to 31° (33°). We will return to the question of overlapping transitions in the concluding section; we state here only the main outcome of our fluorescence study, for it will serve, so to speak, as the peg on which the next section is to be hung: $\theta_{I,III} > \theta_{I,II} > 30^\circ$.

ELD Measurements (Figures 2 and 3). The experiment consists of measuring ELD(0°) and ELD(54.7°), which are defined in eq 2, where $A(\theta')$ denotes the absorbance of the

$$\text{ELD}(0^\circ) = A(0^\circ) - A_r \quad (2a)$$

$$\text{ELD}(54.7^\circ) = A(54.7^\circ) - A_r \quad (2b)$$

sample when the applied electric field makes an angle θ' to the electric vector of the linearly polarized measuring light and A_r is the absorbance in the absence of the electric field (i.e., when the molecules are distributed randomly). According to Labhart (1961), the relations shown in eq 3 enable us to

$$[\text{ELD}(0^\circ) - \frac{2}{3}\text{ELD}(54.7^\circ)] \frac{45k^2T^2}{2\mu^2E^2} = \Delta \quad (3a)$$

$$A_{\parallel\mu} = A_r + (2/3)\Delta \quad (3b)$$

$$A_{\perp\mu} = A_r - (1/3)\Delta \quad (3c)$$

evaluate $A_{\parallel\mu}$ and $A_{\perp\mu}$, absorption parallel and perpendicular to the permanent electric dipole moment μ ; in the relations shown in eq 3, k stands for the Boltzmann constant, T is the temperature, and E denotes the electric field at the molecule.

The angle, Ω , between the direction of the transition moment and the dipole moment can be calculated in accordance with the relation shown in eq 4, provided that μ and E are known;

$$\Omega = \pm \arctan (A_{\perp\mu}/A_{\parallel\mu})^{1/2} \quad (4)$$

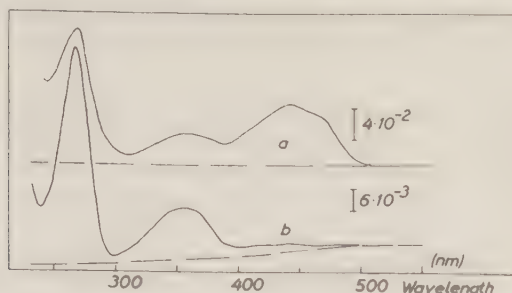


FIGURE 4: (a) Absorption spectrum for FMN solubilized in a lamellar liquid crystal (system A, see text). (b) Corresponding LD for the aligned lamellar mesophase.

unfortunately, this proviso cannot be met for lumiflavin, the molecule subjected to ELD measurement. However, since the angle $\theta_{I,II}$ between the transition moments for the bands I and II is approximately known, it is possible to analyze our data by treating the product $\mu^2 E^2 (= \xi, \text{ say})$ as a variable parameter whose value is to be optimized until agreement with the fluorescence polarization study is obtained, bearing in mind that neither $A_{\parallel\mu}$ nor $A_{\perp\mu}$ can be negative. We found, to our initial dismay, that for $\theta_{I,II} < 39 \pm 4^\circ$ the criterion $A_{\parallel\mu}, A_{\perp\mu} \geq 0$ could not be fulfilled; the value of ξ corresponding to $\theta_{I,II} = 39^\circ$ was therefore considered to be the optimum and led to the results $\Omega_I = \pm 17^\circ$, $\Omega_{II} = \pm 22^\circ$, and $\Omega_{III} = \pm 44^\circ$.

We need now the direction of the dipole moment in the isoalloxazine ring; at present the only course open to us is to use the result of the semiempirical theoretical calculations of Grabe (1972), who found the angle α between the dipole moment and the x_3 axis in Figure 1 to be 75° ; later, independent evidence will be supplied in support of this choice for α . Using her result, we converted the angles Ω into the angles ϕ (measured with respect to the x_3 axis) and arrived at the following values: $\phi_I = 92 \pm 4^\circ$ or $58 \pm 4^\circ$, $\phi_{II} = 97 \pm 3^\circ$ or $53 \pm 3^\circ$, and $\phi_{III} = 119 \pm 2^\circ$ or $31 \pm 2^\circ$. Of the eight possible choices, six can be discounted by invoking the conclusion reached earlier, viz., $\theta_{I,III} > \theta_{I,II} > 30^\circ$, the surviving two sets being $\{\phi_I = 92^\circ, \phi_{II} = 53^\circ, \phi_{III} = 31^\circ\}$ and $\{\phi_I = 58^\circ, \phi_{II} = 97^\circ, \phi_{III} = 119^\circ\}$. We will see shortly that the first set of angles is incompatible with our LD results together with previous data obtained by Eaton et al. (1975). We now state the main conclusion of our study: $\phi_I = 58 \pm 4^\circ$, $\phi_{II} = 97 \pm 3^\circ$, and $\phi_{III} = 119 \pm 2^\circ$.

If we assume that no more than 10% of the absorption of the II and III bands is due to $\pi^* \leftarrow n$ transitions, this would correspond to molar absorptivity contributions of ≤ 800 and $\leq 3000 \text{ M}^{-1} \text{ cm}^{-1}$, respectively. However, this contribution leads only to very small changes ($\sim 2^\circ$) in the angles ϕ_{II} and ϕ_{III} . Thus, we can conclude that the effect of possible $\pi^* \leftarrow n$ transition will not exceed the experimental error of the method.

LD Studies of FMN Solubilized in Macroscopically Aligned Lamellar Liquid Crystals of Monooctanoin and Water (Figure 4). An almost perfect alignment of some lamellar liquid crystals can be obtained by pressing the sample to a thin layer between quartz plates (Johansson et al., 1978). The order parameter of solubilized chromophores in the lamellae can be determined by measuring the LD at an inclined incidence of light relative to the normal of the plates. The order parameter, S , which is a measure of the degree of orientation of the electronic transition moment relative to the director (i.e., the normal of the lamellae), can be obtained from the relation (Johansson et al., 1978) shown in eq 5, where n

$$(LD/A_r)_\omega = 3Sn^{-2}[1 - (n^2 \cos^2 \omega)]^{-1/2} \cos^2 \omega \quad (5)$$

is the refractive index of the lamellar phase and ω is the angle

Table I^a

sample composition monooctanoin- H ₂ O (w/w)	(LD/A _r) _I	(LD/A _r) _{II}	(LD/A _r) _{III}
(A) 90:10	0.016	0.33	0.35
(B) 70:30	-0.016	0.25	0.30
(C) 50:50	-0.021	0.08	0.09

^a $(LD/A_r)_{\omega=45^\circ}$ for bands I, II, and III at three different compositions (A, B, C) of monooctanoin-water lamellar liquid crystals, temperature 23°C .

between the incident light and the plane of the quartz plates.

For a nonsymmetric molecule like the isoalloxazine ring, the measured order parameter, S , can be related to the order parameters of an arbitrarily chosen coordinate system in the molecule (see Figure 1). Thus, $S = \langle D_{00}^{(2)}(\Omega_{DM}) \rangle$ can be transformed to a new molecular frame (M') as follows. Here $D_{00}^{(2)}$ is a Wigner rotation matrix element and Ω_{DM} specifies the Euler angles (α, β, γ) between the molecular (M) and the director frame (D).

For the matrix elements one gets (Brink & Satchler, 1962)

$$\begin{aligned} \langle D_{00}^{(2)}(\Omega_{DM}) \rangle &= \sum_q \langle D_{0q}^{(2)}(\Omega_{DM'}) \rangle \langle D_{q0}^{(2)}(\Omega_{M'M}) \rangle = \\ &= (3/8)^{1/2} \sin^2 \phi [\langle D_{02}^{(2)}(\Omega_{DM'}) \rangle + \langle D_{02}^{(2)}(\Omega_{DM'}) \rangle] + \\ &+ (1/2)(3 \cos^2 \phi - 1) \langle D_{00}^{(2)}(\Omega_{DM'}) \rangle + \\ &+ (3/2)^{1/2} (\sin \phi \cos \phi) [\langle D_{01}^{(2)}(\Omega_{DM'}) \rangle - \langle D_{01}^{(2)}(\Omega_{DM'}) \rangle] = \\ &= S_{11} \sin^2 \phi + S_{33} \cos^2 \phi + 2S_{13} \sin \phi \cos \phi \quad (6) \end{aligned}$$

Here ϕ is the angle between the transition moment and the x_3 axis of the molecular coordinate system $x_1x_2x_3$ of a planar molecule. The order parameters S_{11} , S_{33} , and S_{13} are related to the Wigner matrix elements and

$$S_{33} = \langle D_{00}^{(2)} \rangle = \langle (3 \cos^2 \beta - 1)/2 \rangle$$

$$S_{11} - S_{22} = (3/2)^{1/2} (\langle D_{02}^{(2)} \rangle + \langle D_{02}^{(2)} \rangle) = (3/2) \langle \sin^2 \beta \cos 2\gamma \rangle$$

$$S_{13} = (3/8)^{1/2} (\langle D_{01}^{(2)} \rangle - \langle D_{01}^{(2)} \rangle) = -(3/2) \langle \sin \beta \cos \beta \cos \gamma \rangle$$

Further

$$S_{11} + S_{22} + S_{33} = 0$$

Thus, if there are three different transitions polarized in the molecular plane and the directions of the moments are known (i.e., the angle ϕ), then the order of parameters S_{11} , S_{33} , and S_{13} can be determined. On the other hand, if the transition moments are unknown, the range of possible angles ϕ can be estimated since the order parameters are subjected to certain inequalities: $-1/2 \leq S_{11}, S_{33} \leq 1$, and $-3/4 \leq S_{13} \leq 3/4$. This particular property of the order parameters has been used in our work to distinguish between the different values of the angle obtained from our ELD results (or from other studies as well).

The following lamellar systems, with solubilized FMN, have been studied: (A) 90% w/w monooctanoin, (B) 70% w/w monooctanoin, and (C) 50% w/w monooctanoin.

$(LD/A_r)_{\omega=45^\circ}$ was measured for the I, II, and III bands of FMN for the three different monooctanoin systems, and the results are summarized in Table I. By using eq 5 and 6, the order parameters have been calculated for different angles ϕ for FMN [considering also the data given by Eaton et al. (1975); see below]. These data are summarized in Table II. As can be seen from this table, the only possible combination of the angles is when $\phi_I = 58^\circ$, $\phi_{II} = 97^\circ$, and $\phi_{III} = 119^\circ$,

Table II^a

ϕ_I (deg)	ϕ_{II} (deg)	ϕ_{III} (deg)	A	B	C	order parameter
56	53	119	-1.2	-0.99	-0.38	S_{11}
			-3.1	-2.6	-0.90	S_{22}
			4.3	3.59	1.28	S_{33}
			-0.39	-0.35	-0.13	S_{13}
			0.40	0.28	0.08	S_{11}
58	97	119	-0.20	-0.21	-0.04	S_{22}
			-0.20	-0.07	-0.04	S_{33}
			-0.23	-0.23	-0.08	S_{13}
			0.0	0.0	-0.03	S_{11}
			-1.5	-1.3	-0.42	S_{22}
92	53	119	1.5	1.3	0.45	S_{33}
			-0.11	-0.13	-0.04	S_{13}
			-0.20	-0.20	-0.1	S_{11}
			8.9	7.2	2.6	S_{22}
			-8.7	-7.0	-2.5	S_{33}
92	97	119	-3.1	-2.6	-0.93	S_{13}

^a Order parameters (S_{11} , S_{22} , S_{33} , and S_{13} , where $\sum_{i=1}^3 S_{ii} = 0$) calculated from eq 5 and 6 using the transition moment directions $\{\phi_I, \phi_{II}, \phi_{III}\}$ obtained from the ELD study on lumiflavin. Three different concentrations (A, B, C; see text) of monooctanoin in lamellar liquid-crystalline phase have been used. Note that the results italicized in the table are the only values of the order parameters that are permitted: $-1/2 \leq S_{11}, S_{22}$ and $S_{33} \leq 1$, and $-3/4 \leq S_{13} \leq 3/4$.

since these angles are the only ones that lead to an acceptable set of order parameters.

Discussion

Three optical techniques have been brought to bear on the problem of locating the transition moments for the first three bands in the absorption spectrum of the oxidized form of the isoalloxazine ring. If we calculate relative angles, using the $\{\phi_I, \phi_{II}, \phi_{III}\}$ set finally chosen in the last section, we find $\theta_{I,II} = 39 \pm 4^\circ$ and $\theta_{I,III} = 61 \pm 6^\circ$, values which are not in satisfactory agreement with those of ~ 30 and $\sim 50^\circ$ furnished by the fluorescence study. It is thus natural to inquire into the source(s) of this discrepancy and to assess the reliability of the set of values finally chosen by us. For this purpose, we turn to a comparison of our results with those of some previous studies.

Polarized Spectroscopy of FMN in Single Crystals of Flavodoxin. Eaton et al. (1975) have studied the polarized absorption spectra of FMN in the spectral range covered by bands I and II. They too arrived at a pair of values for each of the angles ϕ_I and ϕ_{II} : $\phi_I = 75 \pm 4^\circ$ or $57 \pm 4^\circ$ and $\phi_{II} = 95 \pm 4^\circ$ or $37 \pm 4^\circ$. They chose the set $\{\phi_I = 75^\circ, \phi_{II} = 95^\circ\}$, eliminating the other three on the basis of (1) linear dichroism studies, conducted by Lhoste (1971), on stretched PVA films impregnated with lumiflavin and (2) a value of $\theta_{I,II} = 20 \pm 5^\circ$ deduced by Sun et al. (1972) from their fluorescence polarization data. As stated in the introductory paragraph, the directions of the transition moments can be unambiguously determined only if the orientational distribution of the chromophore is known; Lhoste's conclusion, made in the absence of this information, is therefore unwarranted. Also, the data reported by Sun et al. (1972) were in the form of measured degrees of polarization rather than the spectroscopically more meaningful intrinsic values. A fresh look at the data published by Eaton et al. (1975) is therefore necessary.

It is evident that if we select, from their data, $\{\phi_I = 57^\circ, \phi_{II} = 95^\circ\}$ as the correct set, the choice would accord well with that made earlier on the basis of our ELD data. One of the reasons offered by Eaton et al. (1975) in support of $\theta_{I,II} = 20^\circ$ was that it led to the value of 79° for θ_{zc} , in excellent agreement

with their experimental value. It is to be noted, however, that $\theta_{I,II} = 38^\circ$ also agrees with the crystallographic data, giving $\theta_{zc} = 78^\circ$.

The reader may recall, however, that our ELD data led us to state that $\phi_I = 92$ or 58° and $\phi_{II} = 97$ or 53° and wonder why, if both papers contain reliable data and their correct choices for the set $\{\phi_I, \phi_{II}\}$ are in such good agreement with each other, the discarded values of ϕ_I and ϕ_{II} in the two papers disagree so conspicuously. Now, for a particular transition each experiment yields two values (equal in magnitude but opposite in sign) for the angle Ω between the transition moment and a reference axis; however, the reference axis in our ELD experiment does not coincide with that used in the single-crystal experiment of Eaton et al. (1975). It follows from elementary geometry that when the angle for a given transition is referred to, say in the x_3 axis, at most one of the two values obtained in one experiment can agree with a value obtained in the other experiment. That such agreement is in fact obtained betokens, in our view, the reliability of both experiments and of Grabe's theoretically calculated value of α .

Photoselection Studies. The study of fluorescence polarization affords, to the wary worker, an exceedingly reliable method for determining the angle θ between the absorption and emission oscillators; however, in spectral characterization of the flavins, uncritical use of the data has been the rule rather than the exception, Weber (1966) being the outstanding exception in our opinion. So as not to be invidious by pointing at the pitfalls committed previously, we will discuss the necessary precautions in relation to our work.

Our first concern was the minimization of instrumental depolarization, hence the decision to dispense with the use of a cryostat and work at room temperature, using glycerol as the solvent. In glycerol, the rotational correlation time of a flavin molecule can be estimated with the aid of the Stokes-Einstein relation to be greater than 100 ns. Fortunately, the first excited state of flavins is quite short-lived, with a lifetime of ~ 5 ns (Gordon-Walker et al., 1970); during its lifetime, the excited molecule can therefore be assumed to be practically immobile, an assumption which will shortly be substantiated on independent grounds.

Our next concern was to see that $p_i(450)$ can indeed be taken as 0.5, which amounts to taking as zero the angle between the emission oscillator and the transition moment for band I, an assumption that has been explicitly and implicitly questioned in the past. We draw the reader's attention to the data published by Chen & Bowman (1965); they studied fluorescein, rhodamine B, and riboflavin under conditions of negligible rotational and concentration depolarization and found that $p(\lambda_1)$ was close to 0.47, where λ_1 ($=450$ nm for riboflavin) denotes a wavelength in the region spanned by the first absorption band. Since the lowest energy absorption band in fluorescein and rhodamine B arises from a strong allowed electronic transition ($\epsilon > 10^5$ M⁻¹ cm⁻¹) with no signs of vibronic interactions, one expects that $p_i(\lambda_1) = 0.5$, an expectation fully borne out by experimental observation (Szalay et al., 1962; Fleming et al., 1976). One may therefore conclude that for FMN, fluorescein, and rhodamine B, in all cases $p(\lambda_1)$ was close to 0.45; for lumiflavin, $p(450)$ was 0.42. Thus, to a very good approximation, excited solute molecules may be regarded as incapable of significant Brownian rotation within their lifetimes, and the introduction of a randomization factor becomes not merely plausible but essential for a sensible analysis of the data.

Gordon-Walker et al. (1970) determined $\theta_{I,II}$ by two photoselection methods: fluorescence polarization and dichroic

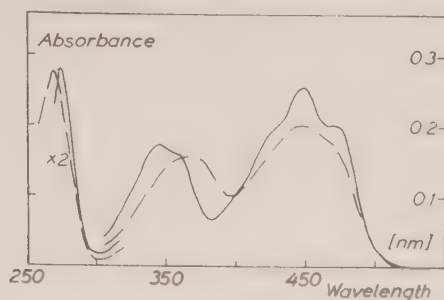


FIGURE 5: Absorption spectra of FMN in glycerol (---) and of lumiflavin in a 1:1 mixture (by volume) of chloroform and carbon tetrachloride (—). The absorption band at 270 nm is diminished by a factor of 2.

bleaching, a method introduced first by Lewis & Bigeleisen (1943). Their fluorescence result, i.e., $\theta_{I,II} = 29 \pm 1^\circ$, is in perfect agreement with ours; more interesting for our purpose is the result of the latter study according to which $\theta_{I,II} = 38 \pm 5^\circ$, in very satisfactory agreement with our ELD results.

The discrepancy between $\theta_{I,II}$ determined from fluorescence studies ($\geq 30^\circ$) and that from dichroic bleaching or ELD studies (38°) is most probably due to solvent shifts. As pointed out by Harbury et al. (1959), the wavelength maximum of band II is strongly dependent on the polarity of the solvent, shifting from ~ 370 nm in water to ~ 330 nm in carbon tetrachloride. Figure 5 displays the absorption spectra of FMN in glycerol, the solvent used in the fluorescence experiment, and of lumiflavin in a 1:1 mixture of chloroform and carbon tetrachloride, the solvent employed for ELD studies; it can be seen that bands I and II overlap to a much larger extent in glycerol than in the mixed solvent. If one makes the not unreasonable assumption that, in glycerol, 7% of the total absorption at 375 nm is contributed by band I, a value of $\theta_{I,II} \approx 38^\circ$ is obtained; further support for this is provided by the observation that the degree of polarization decreases as λ is scanned toward wavelengths shorter than 375 nm. The discrepancy between the two values of $\theta_{I,II}$, 48° deduced from the fluorescence experiment and 61° from the ELD work, is a little more disconcerting and will persist even if allowance is made for the existence of a $\pi^* \leftarrow n$ transition of absorptivity $\leq 3000 \text{ M}^{-1} \text{ cm}^{-1}$. At present we have no satisfactory explanation, but we conjecture that the overlap between band III and the band at 220 nm (band IV) may be responsible for the discrepancy, provided that the latter band is polarized nearly parallel to band I; unfortunately, the position of band IV in the mixed solvent cannot be located due to absorption by the solvent for wavelengths shorter than 260 nm.

Linear Dichroism Studies. The orientation of FMN solubilized in the lamellar liquid-crystalline phase of mono-octanoin and different water concentrations has been studied with LD spectroscopy on macroscopically aligned samples.

From the magnitude of the order parameters (see Table II) it can be inferred that for the samples called A and B one set of angles ϕ can be unambiguously determined. For the system C, where the water concentration is very high, it is not possible to distinguish between the different sets of angles ϕ by using the same argument as for A and B, since all of the order parameters are very small and the inequalities $-1/2 \leq S_{ii} \leq 1$ and $-3/4 \leq S_{ij} \leq 3/4$ can in such cases be satisfied for several sets of the angle. However, one has to demand that for the correct set of angles the order parameters must be acceptable for all three systems. Thus, there is only one choice of the set of angles that can be made, as can be seen in Table II.

Finally, it is interesting to note that the order parameters of FMN in the model membrane are remarkably large. Furthermore, as expected, the orientation of the water-soluble flavin molecule decreases rapidly as the water content in the system increases.

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CORRECTION

One may therefore conclude that $p_i(450)$ for riboflavin must also be equated to 0.5; our results for glycerol solutions of riboflavin, FMN, fluorescein, rhodamine B, and rhodamine 6G are similar to those published by Chen & Bowman (1965)—in all cases, $p(\lambda_i)$ was close to 0.45; for lumiflavin, $p(450)$ was 0.42.

IV

Orientation of β -Carotene and Retinal in Lipid Bilayers.

by

Lennart B-A Johansson, Göran Lindblom, Ake Wieslander* and
Gösta Arvidson**

Division of Physical Chemistry 2
Chemical Center
University of Lund
S-220 07 Lund 7
Sweden

* Dept. of Microbiology, University of Lund, S-223 62 Lund, Sweden

**Department of Physiological Chemistry, University of Uppsala,
Biomedikum, S-751 23 Uppsala, Sweden.

1. Introduction

The function of carotenoids in membranes from photosynthetic bacteria and plants has been investigated intensively during the last thirty years (1-4). In animals certain carotenoids such as β -carotene fulfil an essential physiological function as precursors of vitamin A. By enzymatic oxidation β -carotene is converted to retinol which is further oxidized to retinal i.e. vitamin A.

Although a subject of previous studies (5,6) the positioning of carotenoids in membranes is far from clear. To our knowledge no conclusive experiment has been performed showing the orientation of any carotenoid in lipid bilayers or biological membranes. In the present investigation we have used polarized light spectroscopy and studied the orientation of β -carotene and retinal in different types of lipid bilayers.

2. Experimental

The chromophores all-trans β -carotene and all-trans retinal were from Sigma and EGA-chemie.

The lipids 1-oleoyl-sn-glycerol (monoolein) and polyglycerol monolaurate were from Aktieselskabet Grindstedvaerket Aarhus, Denmark. 1-octanoyl-sn-glycerol (monooctanoin) was synthesized at "Syntestjänst, Chemical Centre, Lund. 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOL) was obtained by acylation of sn-glycero-3-phosphocholine with the fatty acid anhydride as described (7). The mixtures of the chromophore and lipid were prepared by dissolving appropriate amounts of the components (molar ratio of lipid/chromophore $\approx 10^3$) in chloroform, which was then removed by freeze drying. The lamellar liquid crystalline phases of the various lipids were obtained by adding suitable amounts of water and were then left for about two days to reach equilibrium.

The samples were protected from light with aluminium foil during the preparation. The lamellar phases were macroscopically aligned between quartz plates (8, 9). Polarized absorption spectra were recorded with a Varian, Cary 219 supplemented with sheet polarizers (HNP'B, Polarizers, United Kingdom Ltd).

3. Results and Discussion

Lipid bilayers containing small amounts of β -carotene or retinal were macroscopically aligned parallel with the quartz plates. The optical axis of such a uniaxially anisotropic sample coincides with the normal (the so called director) to the quartz plates. By shining polarized light with polarisations forming the angles ω and 90° ($\perp$) with respect to the director the absorbances A_ω and $A_\perp$ are measured. From the dichroic ratio $D_\omega = A_\omega/A_\perp$, information about the molecular orientation of the chromophore can be obtained. An order parameter, S can be calculated (10) from the equation

$$D_\omega = 1 + 3S(1-S)^{-1}n^{-2} \cos^2\omega \quad (1)$$

where n denotes the refractive index of the lamellar phase. The order parameter S describes the average orientation of the chromophore (the electronic transition dipole moment fixed in the molecule) with respect to the director. For example when $S = -0.5$ the transition moment is perpendicular to the director and for $S=1$ it is parallel to it. For an isotropic solution S is equal to zero.

Table I shows the dichroic ratios obtained together with the calculated order parameters. Assuming that the electronic transition moments are parallel to the long axes of the chromophores the following conclusions can be drawn:

1. β -carotene tends to be oriented with its long axis perpendicular to the lipid acyl chains for all the lamellar systems studied.
2. Retinal, on the other hand, is preferentially oriented with its long axis parallel with the hydrocarbon chains.

Our studies clearly show that independently of the bilayer thickness (varying from about 16Å to 40Å), β -carotene (about 25Å long) never spans the lamella. This is not surprising from a physico-chemical point of view since β -carotene is a hydrophobic molecule and therefore prefers to reside within the apolar interior of the membrane. In monooctanoin which forms the thinnest bilayers studied here the order parameter even approaches -0.5 (Table 1). This means that the long axes of the β -carotene molecules are almost parallel to the lamellar surface. Also in thick bilayers the β -carotene molecules tend to adopt this orientation although the ordering is less pronounced than in the thin bilayers. These findings are in contrast to what has been reported by other workers (5,6) cf. Fig. 3 of ref. 6.

Retinal, being amphiphilic, is solubilized in the bilayer with the hydrophilic group anchored at the water-bilayer interface having the long hydrophobic tail directed towards center of the lamellae. It can therefore be expected that carotenoids containing hydrophilic groups at one or both ends may be oriented in a similar way as retinal in the bilayer and they may consequently span the bilayer. Such studies will be reported in a forthcoming paper. Finally we will comment on the suggestion that carotenoids can act as light protecting pigments (4). This idea is supported by our findings, since absorption of light impinging on the membrane is more efficient when the transition dipoles of the light collector are parallel to the plane of the membrane as found for β -carotene. Experimental indications have been reported that the long axes of carotenoids in spinach chloroplasts are parallel to the photosynthetic membranes (11).

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Table The dichroic ratio, $D_{\omega}(\omega=45^{\circ})$, and the calculated orderparameter, S , of β -carotene and retinal in different lamellar liquid crystalline samples at 22°C . The refractive indices used are given within the parenthesis.

System	β -carotene		all-trans-retinal	
	Dichroic ratio, D_{ω}^a	Orderparameter S	Dichroic ratio, D_{ω}^b	Orderparameter S
1-octylmonoglyceride -water; 70-30% w/w	0.78	-0.46 (1.46)	1.26	0.28 (1.48)
Polyglycerol mono- laurate-water; 70-30% w/w	0.89	-0.18 (1.45)	1.31	0.31 (1.46)
1-0leoylmonoglyceride -water; 10-90% w/w	0.94	-0.09 (1.47)	1.20	0.23 (1.51)
Dioleoylphosphatidyl- choline-water; 80-20% w/w	0.87	-0.22 (1.45)	1.21	0.23 (1.47)

- a. At 460 nm; Exp. accuracy of D_{ω} is ± 0.01
b. At 380 nm; Exp. accuracy of D_{ω} is ± 0.01

V

STUDIES OF CHROMOPHORES IN MODEL MEMBRANES BY POLARIZED
LIGHT ABSORPTION SPECTROSCOPY. The orientation and binding
of tetracaine and procaine.

by

Lennart B.-Å. Johansson and Göran Lindblom
Physical Chemistry 2, Chemical Centre, University of Lund
P.O.Box 740, S-220 07 LUND, Sweden

Running title: orientation of tetracaine in bilayers.

ABSTRACT

A method for studying the orientation and binding of chromophores in macroscopically aligned membranes by polarized light absorption spectroscopy is described.

Here tetracaine and procaine solubilized in a lamellar phase of octyl-1-glyceride(monooctanoin) and water have been investigated.

Tetracaine is found to be located in the lipid region with a preferential orientation of the molecular long axis parallel to the hydrocarbon chains. The orientation of procaine, mainly residing in the water region, is very small.

INTRODUCTION

There has long been a great interest in the physico-chemical properties of membrane-active drugs in lipid bilayers. This pertains in particular to local anesthetics which are drugs that reversibly block the action potential of the nerve or muscle cell without appreciably affecting the resting membrane potential of the cell. Recent reviews on the membrane actions have been written by Seeman (1); Papahadjopoulos (2) and a book edited by Fink (3) is available. Because local anesthetics like tetracaine and procaine have surface active properties they are thought to function through interactions with the membrane lipids. Such a lipid theory was originally proposed by Overton (4) and Meyer (5) emphasizing the predominant role of lipid solubility of the noncharged form of the anesthetic. Skou (6) also demonstrated a correlation between the potency of local anesthetics in blocking nerve conduction and their penetration into lipid monolayers.

Previously nuclear magnetic resonance methods have also been used to investigate the interaction of local anesthetics with membrane lipids. However contradictory results have been reported since Hauser et al (7) concluded from their findings that no interaction occur between local anesthetics and zwitterionic phosphatidylcholine in the absence of a net negative charge. The interaction was therefore thought to be mainly electrostatic. Fernández and Cebrón (8) on the other hand showed that tetracaine interacts hydrophobically with zwitterionic lecithin while procaine did not.

In order to get further insight into the interaction of anesthetics with lipid membranes we have used a polarized absorption spectroscopical method (linear dichroism) to investigate the binding and molecular orientation of tetracaine and procaine in model membranes composed of a non charged lipid, monooctanoin. Light spectroscopy is particular useful for such studies since almost all anesthetics contain rather

strong chromophores. The molecular orientation of the chromophore in the membrane can be determined from measurements of the linear dichroism on a macroscopically aligned sample. In a recent review (9) the theoretical treatment of polarized light spectroscopy on membrane systems have been given together with a critical discussion of the use of the methods to obtaining the molecular orientation.

METHOD

The absorbance (A) which arise from the interaction of the electric transition dipole moment ($\vec{p}$) and the electric field vector ($\vec{E}$) of weak electromagnetic radiation is given by

$$A = k(\vec{p} \cdot \vec{E})^2 \quad (1)$$

where k is a constant.

The transition moment is regarded as a vector fixed in the molecule and the vector, $\vec{E}$ defines the linear polarization of light in a laboratory fixed coordinate system. In order to perform the scalar product in equation (1) the transition moment is transformed from the molecular frame (M) to the laboratory coordinate system (L). This is accomplished most conveniently by first mathematically forming a tensor $\tilde{P}$ by a direct product between two p-vectors:

$$\tilde{P} = \vec{p} \otimes \vec{p} = \begin{pmatrix} p_x^2 & p_x p_y & p_x p_z \\ p_x p_y & p_y^2 & p_y p_z \\ p_x p_z & p_y p_z & p_z^2 \end{pmatrix} \quad (2)$$

The absorbance of a sample can then be written

$$A = k \vec{E} \tilde{P} \vec{E} \quad (3)$$

Representing the vector $\vec{E}$ by a (1×3) matrix, $\underline{E}$, and the tensor $\tilde{P}$ by a (3×3) matrix, $\underline{P}$, equation (3) becomes

$$A = k \underline{E}^L \underline{P}^L \underline{E}^{L,t} \quad (4)$$

where t stands for the transpose. The transformation of $\underline{P}^M$ to $\underline{P}^L$ can, of course, be performed by using a Cartesian transformation tensor, although somewhat cumbersome. Here we will instead use irreducible spherical tensors, since the different tensor components, P_{+2} , P_{+1} and P_0 , have simple transformation properties under rotation of the coordinate system. The transformation of the spherical tensor elements P_m^M to an element P_n^L is given by

$$P_n^L = \sum_{m=-2}^2 D_{nm}^{(2)*}(\Omega) P_m^M \quad (5)$$

$D_{nm}^{(2)*}$ is a Wigner rotation matrix element of the eulerian angles $\alpha, \beta, \gamma \equiv \Omega$ (10). For systems having a k -fold ($k > 2$) symmetry axis (often called the director), the distribution density function is

$$f(\alpha, \beta, \gamma) = f\left(\alpha + \frac{2h\pi}{k}, \beta, \gamma\right) = f(\beta, \gamma) \\ h = 1, 2, \dots, k \quad (6)$$

The uniaxial space averages (denoted by $\langle \rangle$) of Equation (5) is therefore

$$\langle P_n^L \rangle = \sum_{m=-2}^2 \langle D_{nm}^{(2)*}(\beta, \gamma) \rangle P_m^M \quad (7)$$

By using the relation between the five irreducible components (P_n^L) and the nine cartesian components we obtain the following absorptivity tensor

$$\underline{P}^L = \begin{pmatrix} \frac{1}{3} \text{Tr} \tilde{P} - \frac{1}{3} \sum_n \langle D_{on}^{(2)*}(\beta, \gamma) \rangle P_n^M & 0 & 0 \\ 0 & \frac{1}{3} \text{Tr} \tilde{P} - \frac{1}{3} \sum_n \langle D_{on}^{(2)*}(\beta, \gamma) \rangle P_n^M & 0 \\ 0 & 0 & \frac{1}{3} \text{Tr} \tilde{P} + \frac{2}{3} \sum_n \langle D_{on}^{(2)*}(\beta, \gamma) \rangle P_n^M \end{pmatrix} \quad (8)$$

where the Z axis is chosen parallel to the symmetry axis of the system. Consider a system in which a mole fraction, p , is in an anisotropic (uniaxial) site, while the remaining fraction, $1-p$, is in an isotropic site. The distribution density function of the system then becomes

$$f'(\beta, \gamma) = (1-p) \frac{1}{8\pi^2} + p f(\beta, \gamma) \quad (9)$$

Applying this distribution density one obtains

$$\langle D_{om}^{(2)*}(\beta, \gamma) \rangle \equiv \int_{\beta\gamma} f'(\beta, \gamma) D_{om}^{(2)*}(\beta, \gamma) \sin \beta d\beta d\gamma = p \langle D_{om}^{(2)*}(\beta, \gamma) \rangle \quad (10)$$

For such a system the absorptivity tensor is

$$\langle P^L \rangle = \begin{pmatrix} \frac{1}{3} \text{Tr} \tilde{P} - \frac{1}{3} \sum p \langle D_{on}^{(2)}(\beta\gamma) \rangle^* P_n^M & 0 & 0 \\ 0 & \frac{1}{3} \text{Tr} \tilde{P} - \frac{1}{3} \sum_n p \langle D_{on}^{(2)}(\beta\gamma) \rangle^* P_n^M & 0 \\ 0 & 0 & \frac{1}{3} \text{Tr} \tilde{P} + \frac{2}{3} \sum_n p \langle D_{on}^{(2)}(\beta\gamma) \rangle^* P_n^M \end{pmatrix} \quad (11)$$

The absorbances with the polarizations of light perpendicular ($A_{\perp}$) and at an angle (A_{ω}) with the director (See Fig. 1) yield

$$A_{\perp} = k (0 \ 1 \ 0) \langle P^L \rangle (0 \ 1 \ 0)^t = k \left(\frac{1}{3} \text{Tr} \tilde{P} - \frac{1}{3} \sum_n p \langle D_{on}^{(2)}(\beta\gamma) \rangle^* P_n^M \right) \quad (12)$$

$$\begin{aligned} A_{\omega} &= k (-\sin \omega \ 0 \ \cos \omega) \langle P^L \rangle (-\sin \omega \ 0 \ \cos \omega)^t = \\ &= k \left(\sum_n p \langle D_{on}^{(2)}(\beta\gamma) \rangle^* P_n^M \cos^2 \omega + \frac{1}{3} \text{Tr} \tilde{P} - \frac{1}{3} \sum_n p \langle D_{on}^{(2)}(\beta\gamma) \rangle^* P_n^M \right) \end{aligned} \quad (13)$$

The molecular orientation is obtained from the linear dichroism, $LD \equiv A_{\omega} - A_{\perp}$, and $A_{\perp}$ according to Equation (14).

$$\frac{LD}{A_{\perp}} = \frac{3 \sum_n \langle D_{on}^{(2)*}(\beta\gamma) \rangle P_n^M p \cos^2 \omega}{\text{Tr} \tilde{P} - \sum_n \langle D_{on}^{(2)*}(\beta\gamma) \rangle P_n^M \cdot p} \quad (14)$$

If the molecular z-axis is chosen along the transition moment, n is equal to zero in equation (14). Thus

$$\frac{LD}{A_{\perp}} = \frac{3 \langle D_{00}^{(2)*}(\beta) \rangle p \cos^2 \omega}{1 - \langle D_{00}^{(2)*}(\beta) \rangle p} = \frac{3pS}{1-pS} \cos^2 \omega \quad (15)$$

where $S = \langle D_{00}^{(2)*}(\beta) \rangle$ is the more familiar order parameter introduced by Saupe (12).

EXPERIMENTAL

Absorption spectra were recorded on a Varian Cary 219 absorption spectrometer. The linear dichroism, LD, was measured on a home-built apparatus utilizing a Jobin Yvon HRS2 monochromator and a Jobin Yvon photoelastic modulator.

Monoctanoin was purchased from "Syntestjänst" Lund University; procaine and tetracaine was bought from Sigma. The samples were macroscopically aligned between quartz plates according to a procedure previously reported (12, 13). All investigation was performed at pH equal to about 6.

RESULTS AND DISCUSSION

Tetracaine (hydrochloride) in water absorb light with a maximum at about 310 nm having a molar absorptivity determined to be $2.3 \cdot 10^4 \text{ mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}$. It can be assumed that this absorption is due to a $\pi \leftarrow \pi^*$ transition polarized in the plane of the phenyl ring and directed along the parasubstitution of the ring (cf. Fig. 2). The $\pi \leftarrow \pi^*$ assumption

is supported by the red shift obtained for tetracaine (uncharged, free base) when dissolved in solvents with increasing polarity as shown in Table 1, where $\lambda_{\max}$ of the drug in hexane, ethanol and water is given.

Knowing the direction of the transition dipole in the molecule the orientation of tetracaine in a macroscopically aligned sample can be studied by polarized absorption spectroscopy. Such a method has been used here to study the orientation of the drug solubilized in a model membrane composed of octyl-1-glyceride, monoctanoin, and water. The absorption of light was detected with the macroscopically aligned sample at an angle of $\pi/4$ between the normal of the quartz plates and the direction of propagation of light as depicted in Fig. 1. A detailed discussion of this technique has been reported previously (9). The results obtained are shown in Fig. 3, where $LD/A_{\perp}$ is plotted as a function of the molar ratio between monoctanoin and tetracaine. It is clear from this Figure that $LD/A_{\perp}$ for the charged tetracaine is constant at all concentrations varying over more than one order of magnitude. On the other hand for the tetracaine free base, $LD/A_{\perp}$ is lower than that of tetracaine at high concentration but it approaches the constant value obtained for tetracaine at lower concentration of the drug. These experimental findings can be interpreted by adopting the two site model considered in the METHOD section with the following two sites; 1) molecules residing in an anisotropic environment within the lipid bilayer, having an order parameter S and 2) molecules in the water layer subjected to an isotropic orientational distribution giving rise to an order parameter equal to zero. The mole fraction of molecules in the anisotropic site is p . The order parameter can then be obtained from

$$\frac{LD}{A_{\perp}} = 3pS(1-pS)^{-1} n^{-2}(1-n^{-2}\cos^2\omega)^{-\frac{1}{2}}\cos^2\omega \quad (16)$$

where also n , the refractive index of the lamellar sample has been taken into account (15). Since $n=1.5$ (14) and $\omega=\pi/4$ the constant value of the apparent order parameter, pS , of tetracaine can be calculated to be 0.24 ± 0.02 . The lowest

value of pS for tetracaine free base is equal to 0.15. The positive order parameter ($p > 0$) for both tetracaine and tetracaine free base implies that the long axes of the molecules are preferentially oriented parallel to the hydrocarbon chains.

Since the tetracaine molecule is amphiphilic (see fig. 2) with a rather long hydrophobic part, a constant pS over a wide concentration range strongly indicates that the drug is solubilized within the lipid bilayer. This is further supported by the rather high value of $pS=0.24$. In previous studies (see ref. 9) we have found that the order parameters of long chromophores solubilized in monoctanoin are in general around 0.1-0.3, e.g. for retinal $S=0.28$. These observations taken together strongly indicate that most of the tetracaine molecules are located in the lipid bilayer, i.e. p is close to unity. This interpretation is further supported by the findings obtained for the free base. For this molecule $LD/A_{\perp}$ is the same as for tetracaine at low concentrations but decreases at higher concentrations. This means that either p and/or S diminish with increasing free base content. Since this molecule is hydrophobic it may be located also in the interior of the membrane where the molecular ordering is lower as has been shown by deuterium NMR studies (16). With increasing concentration of this drug it may therefore be expected that an increasing amount of the molecules have to be in the middle of the lipid bilayer. Hence the most probable explanation of the decrease observed in $LD/A_{\perp}$ is due to the lower molecular ordering of molecules at the center of the bilayer. This conclusion is supported by the following experimental finding shown in Table II. An increase in the content of tetracaine free base leads to a shift of the absorption maximum to a shorter wave length, while for tetracaine the $\lambda_{\max}$ does not vary with the concentration. Thus at higher concentrations higher amounts of tetracaine free base will be located in a more hydrophobic milieu. (cf. Table I). For tetracaine, on the other hand, the solubilization site will not change with concentration.

For procaine the LD was found to be very small. The order parameter calculated after correction for multiple reflection (14) was negative and very small, in ^{the} order of 10^{-2} . The observation of a slight decrease in the order parameter with increasing procaine content indicate that procaine is mainly located in the water layer.

CONCLUSIONS

It has been found that the drug tetracaine is completely solubilized with an order parameter equal to 0.24 in the lipid membrane. Procaine, on the other hand, was found to reside in the water layer with a very small order parameter. The method used here could be generally applied for studies of the binding and orientation of chromophoric molecules in macroscopically aligned samples.

ACKNOWLEDGEMENT

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Table I. Absorption maxima of tetracaine in three different solvents.

Solvent	Chromophore	$\lambda_{\max}/\pm 1 \text{ nm}$
n-hexane	tetracaine	
	free base	292
	tetracaine	- a
ethanol	tetracaine	
	free base	308
	tetracaine	311
water	tetracaine	
	free base	309
	tetracaine	311

a) Solubility less than $10^{-6} \text{ mol dm}^{-3}$ and could therefore not be observed.

Table II. Absorption maxima of tetracaine solubilized to various amounts in the lamellar phase composed of monoctanoin and water.

Molar ratio C_8 -monoglyceride / anesthetic	$\lambda_{\max} (\pm 1 \text{ nm})$ Tetracaine free base	$\lambda_{\max} (\pm 1 \text{ nm})$ Tetracaine
$4.33 \cdot 10^3$	313	
$2.16 \cdot 10^3$	312	310
$1.62 \cdot 10^3$		310
$1.08 \cdot 10^3$	312	310
$4.32 \cdot 10^2$	312	310
$2.16 \cdot 10^2$	311	310
$1.44 \cdot 10^2$		310
$1.08 \cdot 10^2$	310	310
$7.2 \cdot 10$	309	

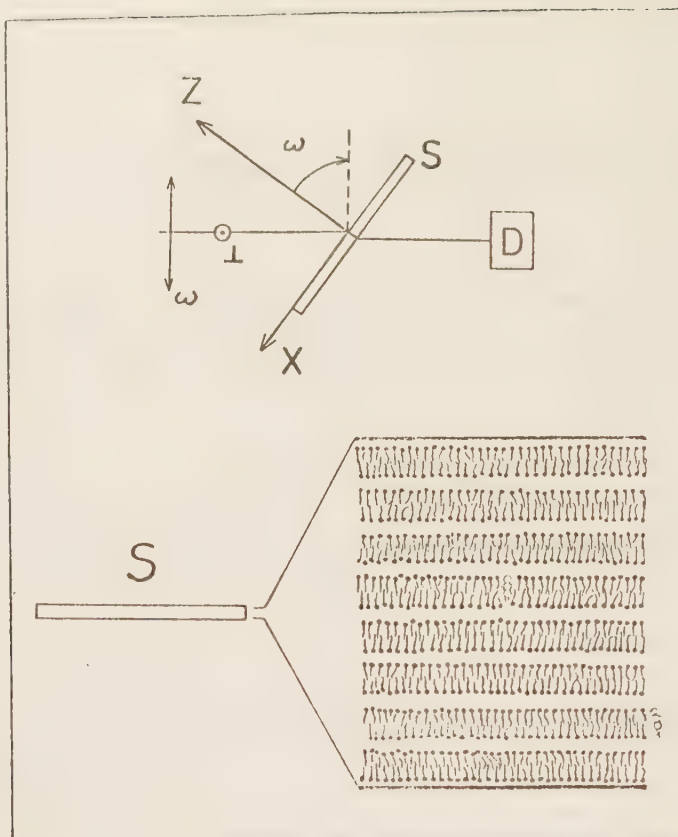


Fig. 1. Polarized absorption measurement: Light with the polarization directions either ω or $\perp$ impinges on an aligned lamellar sample (S). The transmitted light is detected at D.

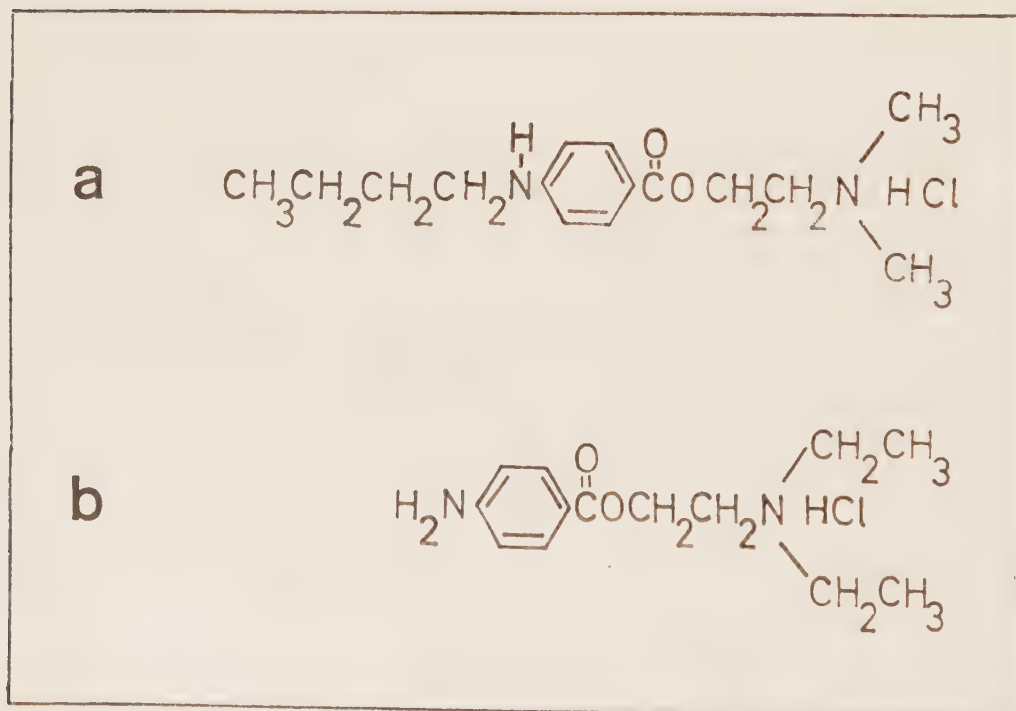


Fig. 2. Structure formulas of a. tetracaine and b. procaine.

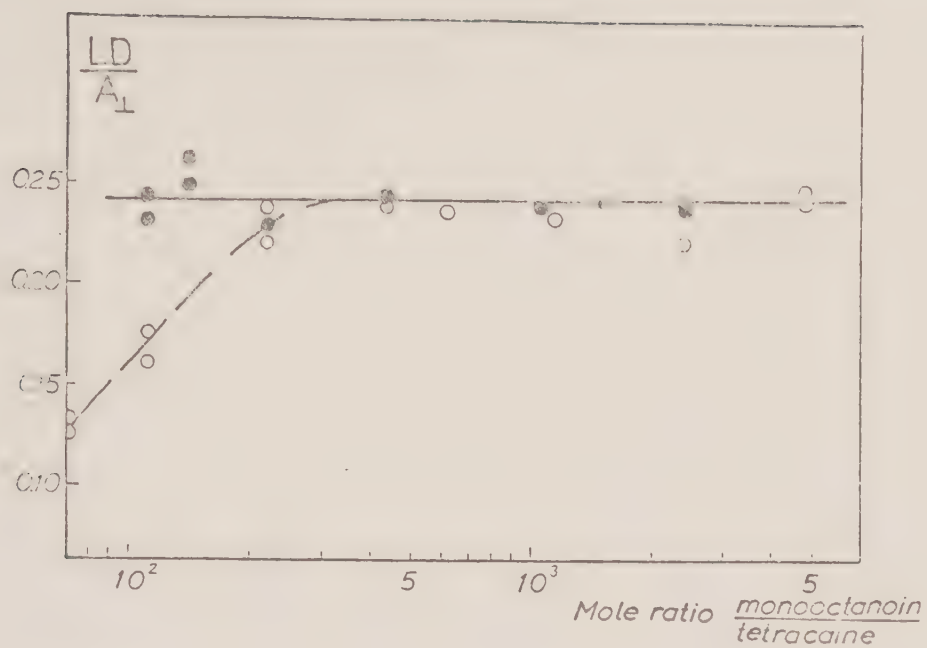


Fig. 3. The ratio between the linear dichroism, LD, and the absorption perpendicular to the quartz plates, $A_{\perp}$, as a function of the molar ratio between mono-octanoin and tetracaine (●) and as a function of the molar ratio between mono-octanoin and tetracaine free base (○). The temperature was 22°C.

VI

The Structure of a Lyotropic Liquid Crystalline Phase that Orients in a Magnetic Field

OLLE SÖDERMAN, GÖRAN LINDBLOM, LENNART B.-Å. JOHANSSON
and KRISTER FONTELL

*Department of Physical Chemistry 2, Chemical Center,
University of Lund, S-220 07 Lund 7, Sweden*

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The structure of a lyotropic liquid crystalline phase with positive diamagnetic anisotropy (type I), that spontaneously orients in a magnetic field has been studied by means of water NMR quadrupole splittings, NMR diffusion and polarized absorption spectroscopy. It is concluded that this phase is built up of long rodlike aggregates. A preliminary study of a sample with negative diamagnetic anisotropy (type II) shows that this phase probably consists of lamellar aggregates. It is suggested that these phases are suitable as orientation matrices for studies of chromophores with polarized light spectroscopy.

INTRODUCTION

About ten years ago Lawson and Flautt^{1,2} discovered that some lyotropic liquid crystalline phases are able to orient spontaneously in a magnetic field when an electrolyte is added. Since then several studies of this type of systems have been reported.^{3–6} The most important application of such phases is to use them as order matrices for solute molecules for nuclear magnetic resonance (NMR) studies.^{3–5} These phases have the great advantage compared with, e.g. thermotropic liquid crystalline systems, that both lipid and water soluble substances can be conveniently dissolved in the system. Usually thermotropics have high solubility for hydrophobic compounds only. Although these lyotropic systems have been utilized and studied extensively, their phase structure has not yet been fully established.

In this communication we have attempted to determine the structure for one of these lyotropic mesophases that orient in a magnetic field. The

samples studied have positive diamagnetic anisotropy (type I).⁶ We have utilized several spectroscopic techniques such as deuterium NMR, pulsed NMR for diffusion studies and polarized absorption spectroscopy.

EXPERIMENTAL

The samples studied contained sodium decylsulfate, sodium sulfate and heavy water. Their compositions were taken from Ref. 6 and the method of preparation was the same. The samples were macroscopically aligned in two ways; (i) by placing the sample tube in a magnetic field, (ii) by pressing the sample between glass plates.⁷ It should be noted that in the first alternative the samples kept the orientation for several hours after being removed from the magnetic field.

²H NMR spectra were recorded at 15.351 MHz on a Varian XL 100 NMR spectrometer operating in the FT mode. To obtain a typical spectrum usually about 500 transients were accumulated.

The amphiphile diffusion studies were performed with a technique based on a standard NMR method⁸ for measurements of diffusion coefficients in systems, where high resolution NMR spectra are observed. Normally such spectra are not obtained for an anisotropic liquid crystalline system. However, by a macroscopical alignment of the mesophase, high resolution spectra can be observed by orienting the sample so that the director (the symmetry axis) and the magnetic field direction make an angle of 54.7° (the "magic angle"). With such an oriented sample the conventional pulsed NMR technique with pulsed magnetic field gradients may be used to determine the diffusion coefficients.^{9,10} The experimental equipment was a Bruker 322s pulsed NMR spectrometer supplemented with a homebuilt pulsed magnetic field gradient unit. The diffusion coefficient is obtained from the attenuation, E_g/E_0 , of a spin echo by varying the strength of the magnetic field gradients (δ or g) or the distance, Δ , between them according to the relation derived by Stejskal and Tanner⁶

$$\ln \frac{E_g}{E_0} = (\gamma g \delta)^2 D \left(\Delta - \frac{\delta}{3} \right)$$

where δ is the width and g the height of the magnetic field gradients, γ is the gyromagnetic ratio and D is the diffusion coefficient. Typical settings in an experiment are $\Delta = 15$ ms, $\delta = 2$ ms, while g varies between 0 and 2.5 T m^{-1} . All measurements were performed relative to a glycerol reference with a known diffusion coefficient.¹¹ The probe temperature was 28°C throughout. Polarized absorption spectra were obtained from samples that had been kept in a magnetic field for at least 12 h. These mesophases contained a small

amount of dissolved retinal. The spectrometer used was a Varian, Cary 219. The absorption spectra were recorded with the linear polarizers parallel ($A_{\parallel}$ mode) and perpendicular ($A_{\perp}$ mode) to the magnetic field, **B**. Polarized absorption was also recorded for retinal in some lamellar liquid crystals aligned between glass plates.

RESULTS AND DISCUSSION

Water ^2H NMR studies

The orientation in a magnetic field of a sample with the composition 39.8% sodium decylsulphate, 4.5% Na_2SO_4 and 55.7% $^2\text{H}_2\text{O}$ (weight percentage) was monitored by observing the change of the deuteron quadrupole splitting with time. For a fully oriented sample the deuteron NMR spectrum consists of two equally intense peaks with the separation $\Delta(\theta)$, the quadrupole splitting, given by^{12,13}

$$\Delta(\theta) = |\sum p_i v_Q^i S_i (3 \cos^2 \theta_{LD} - 1)| \quad (1)$$

where p_i is the fraction of deuterons in site **i**, $v_Q^i = \frac{3}{4}\chi$ (χ is the quadrupole coupling constant), S_i is an order parameter describing the average orientation of the water molecules in the site **i** with respect to the director and θ_{LD} is the angle between the z -axes of the laboratory and the director coordinate systems.

For a nonoriented (powder) spectrum the quadrupole splitting becomes:

$$\Delta = |\sum p_i v_Q^i S_i| \quad (2)$$

Figure 1 illustrates how the ^2H NMR spectrum changes with time. From this figure it can be seen that the initial spectrum has a shape typical of a powder sample. The spectrum gradually changes with time to a shape typical of an oriented sample having two sharp peaks. If the oriented sample is rotated 90° around the NMR tube axis the spectrum in Figure 2 is obtained. In this spectrum the quadrupole splitting is reduced to one half of its original value before the sample was rotated. Equation 1 then shows that the director of the oriented sample lies parallel with the applied magnetic field, **B**.

Samples were also macroscopically aligned between glass plates. Some typical NMR spectra obtained at different discrete angles between the normal to the glass plate and the magnetic field are shown in Figure 3. It can be inferred from this figure that the NMR spectra have characteristic shapes at different angles. Similar spectra have recently been obtained for a hexagonal phase aligned between glass plates.^{14,15} From a simulation of these spectra it

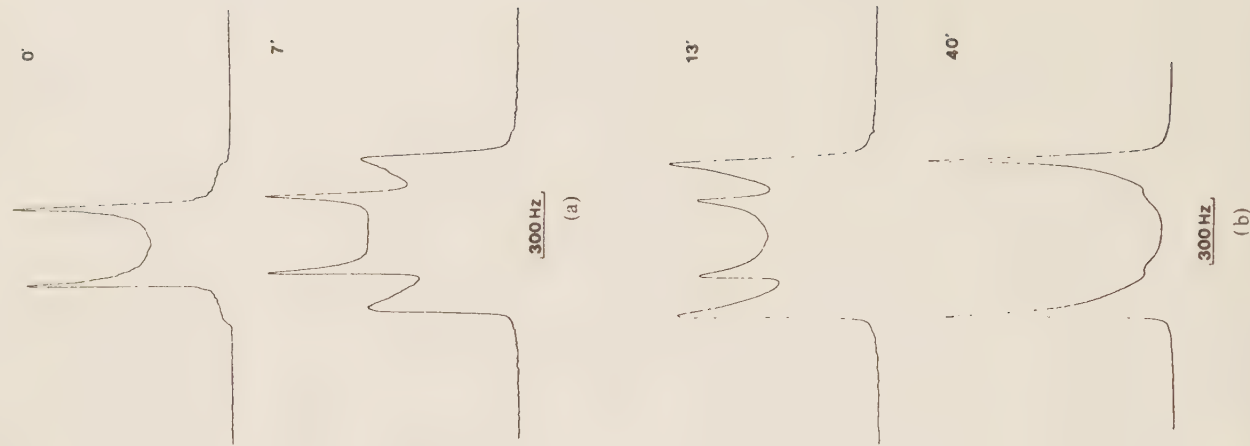


FIGURE 1 ^2H NMR spectra of $^2\text{H}_2\text{O}$ at different time intervals, going from a typical powder pattern at 0' and ending with an oriented sample after 40' in the magnet. All spectra are for a type I sample.

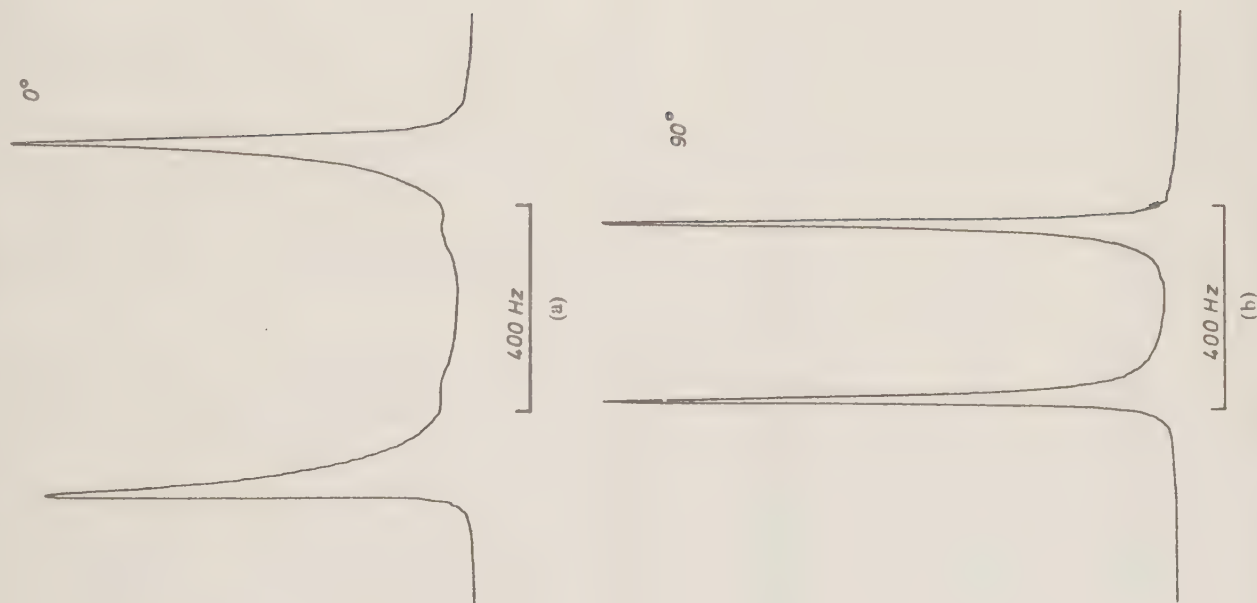


FIGURE 2 ^2H NMR quadrupole splittings of $^2\text{H}_2\text{O}$ for a type I sample, oriented with the director parallel (a) and perpendicular (b) to the magnetic field after a rotation of the oriented sample by 90° . The difference in the splittings obtained is equal to 2.

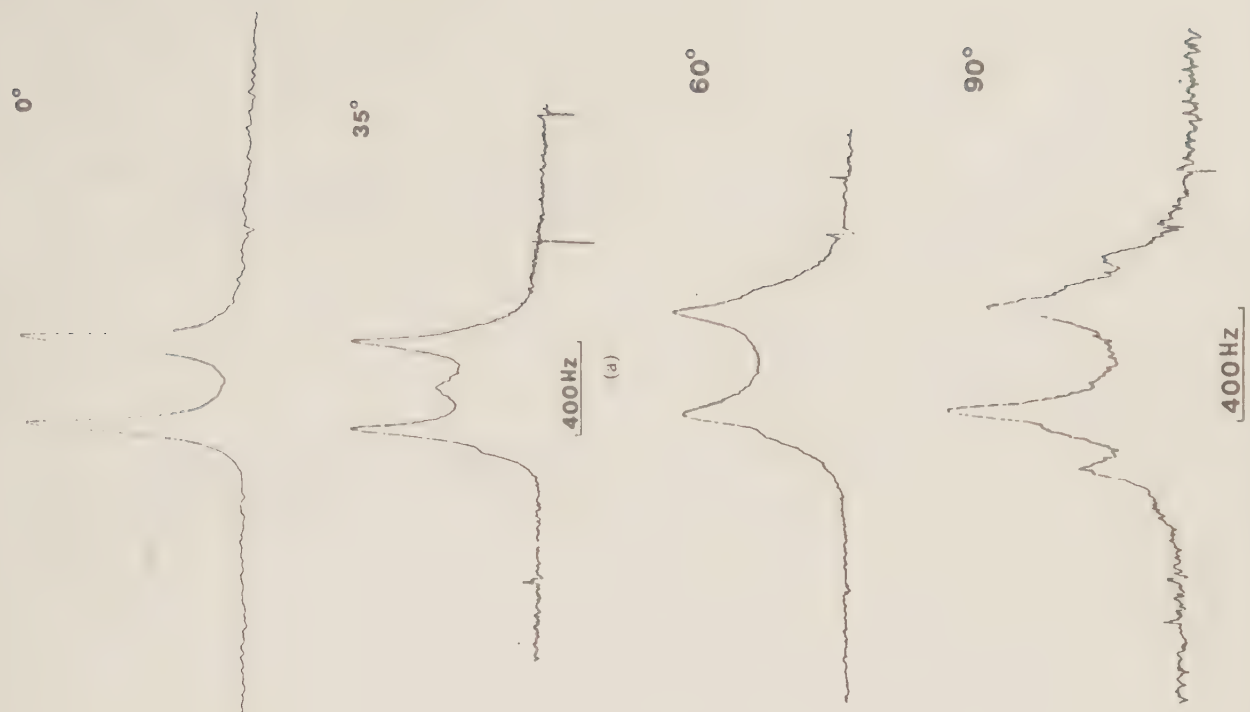
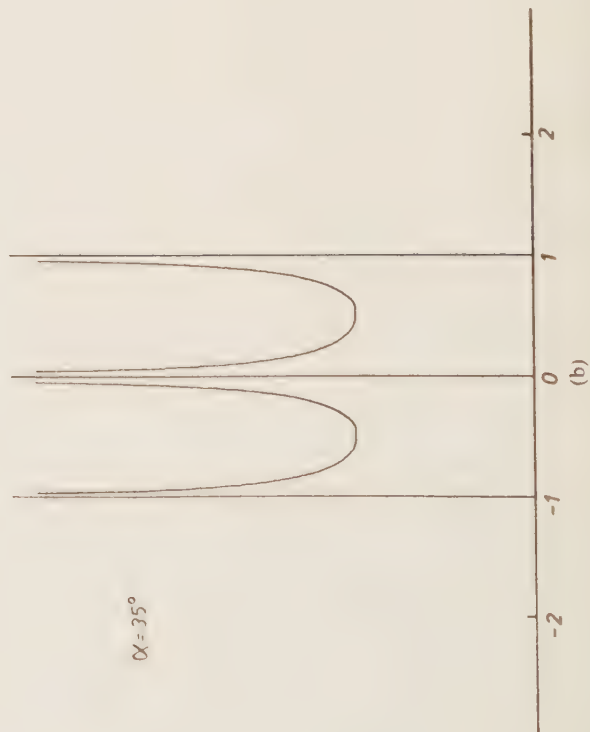
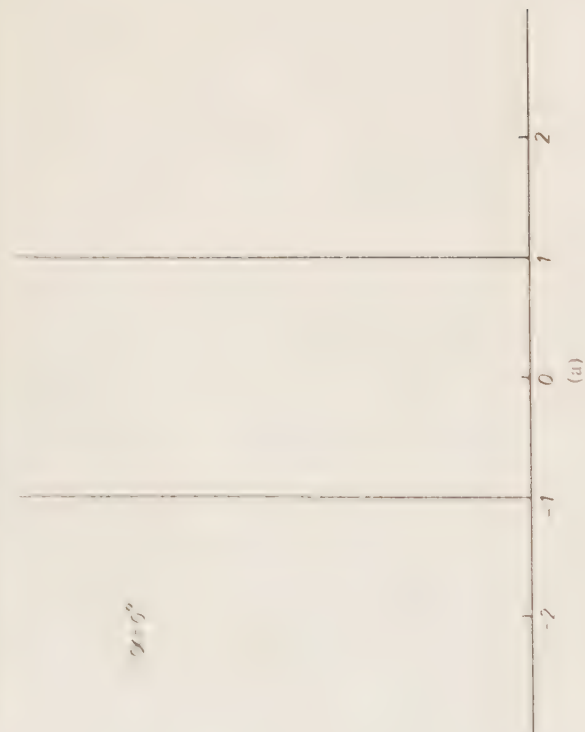
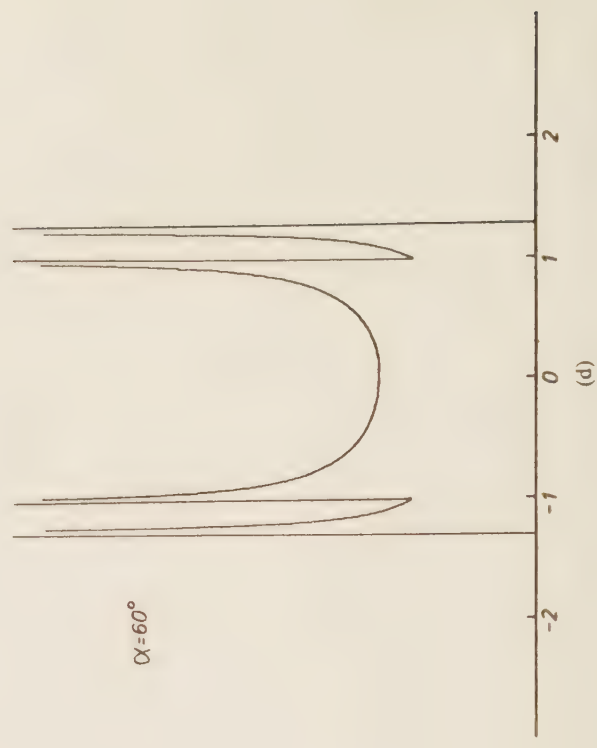
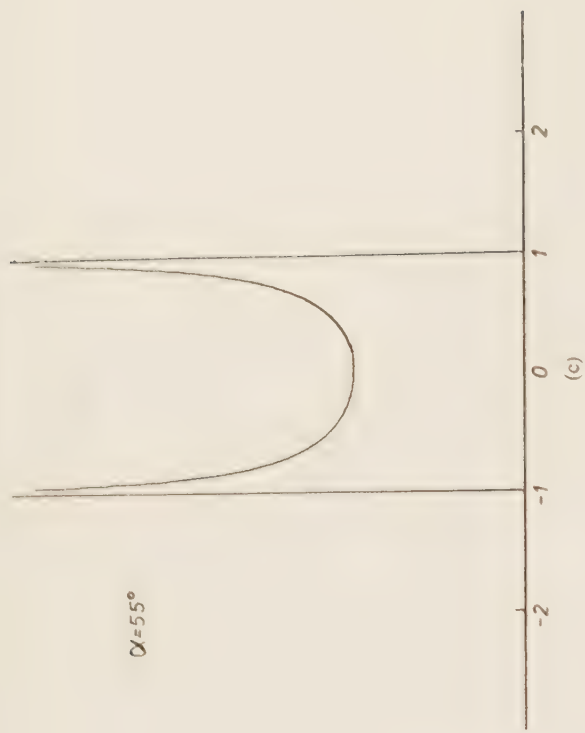


FIGURE 3 ^2H NMR quadrupole splittings for a type I sample macroscopically aligned between glass plates and oriented at different approximate angles in the magnetic field.

FIGURE 4 *continued*FIGURE 4 *continued*

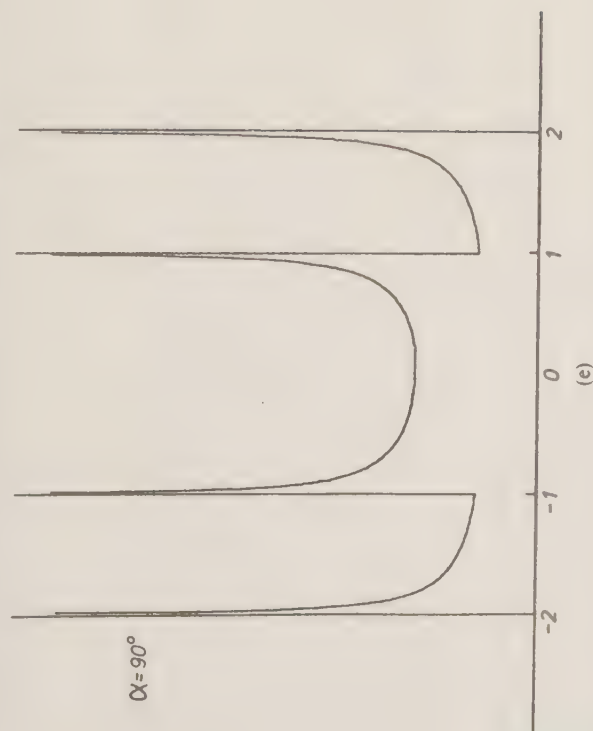


FIGURE 4 Simulated spectra for a sample having its directors randomly oriented in the plane of the glass plates. The glass plates are oriented at different angles α in the magnetic field as in Figure 3 (see text for details). The frequency scale is in units of $4\nu/3\gamma \cdot S$.

was concluded that the director is randomly oriented in the plane of the glass plates. We have simulated spectra with the director randomly oriented in a plane, using the equations for the line shape $g(\nu)$ as derived by Wennerström.¹⁶

$$g_+(x) = \left(\frac{1+x}{3} \right)^{-1/2} \left(\sin^2 \alpha - \frac{1+x}{3} \right)^{-1/2}; \quad -1 \leq x \leq 3 \sin^2 \alpha - 1$$

$$g_-(x) = \left(\frac{1-x}{3} \right)^{-1/2} \left(\sin^2 \alpha - \frac{1-x}{3} \right)^{1/2}; \quad 1 - 3 \sin^2 \alpha \leq x \leq 1 \quad (3)$$

$$g(x) = g_+(x) + g_-(x)$$

where $x = 4\nu/3\gamma S$ and α is the angle of rotation of the plates in the magnetic field. Figure 4 shows spectra calculated from the above equations for some discrete α -values.

Thus Figures 3 and 4 strongly indicate that the director is randomly oriented in the plane of the glass plates. A similar preliminary study of samples with negative diamagnetic anisotropy (type II) (sodium decylsulfate, sodium sulfate, decanol, heavy water) suggests that in this case the director is perpendicular to the glass plates (Figure 5).

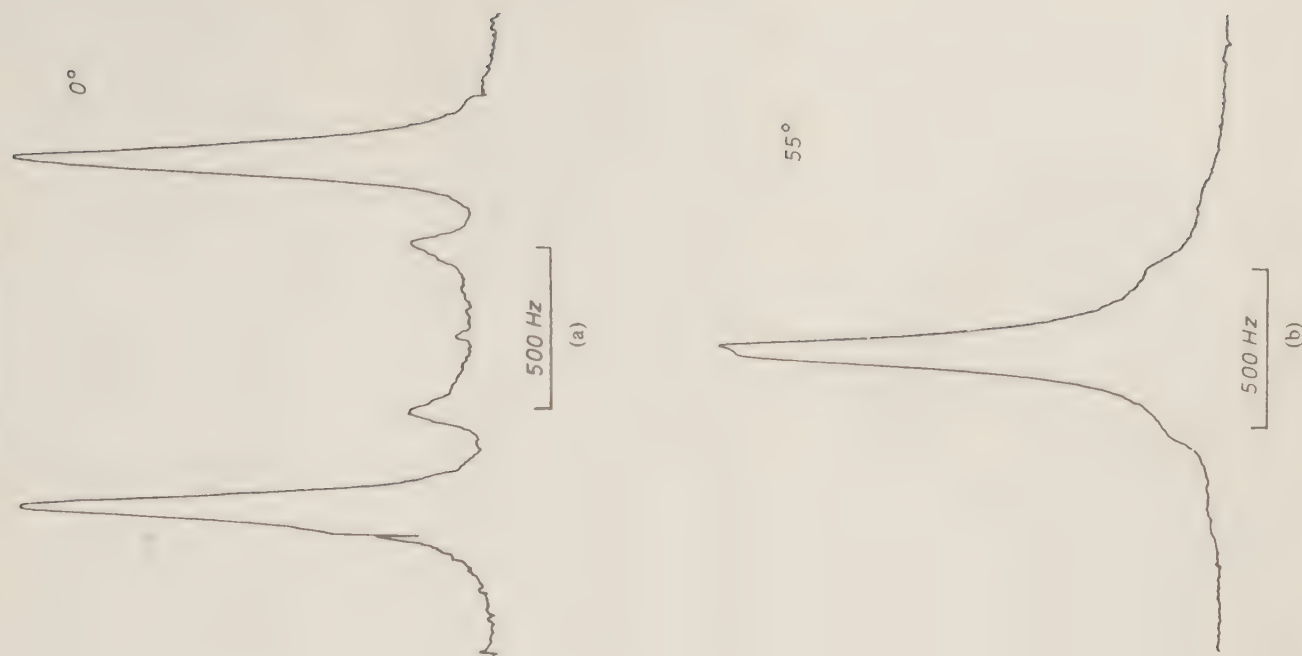


FIGURE 5 *continued*

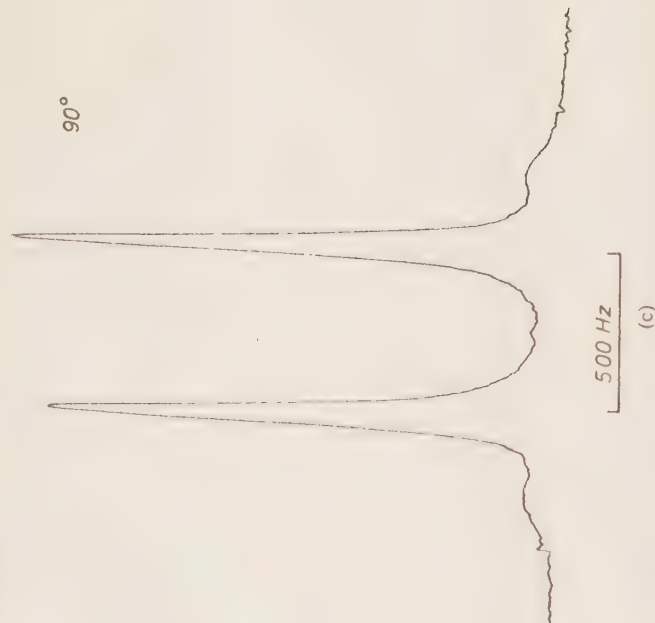


FIGURE 5 ^2H NMR splittings of $^2\text{H}_2\text{O}$ for a type II sample macroscopically aligned between glass plates. These spectra are in agreement with a lamellar phase aligned parallel to the glass plates

Polarized absorption studies

In order to gain further information on the structure of the amphiphilic aggregates building up the type I phase we have also performed polarized absorption studies of the orientation of a solubilized hydrophobic chromophore, retinal. The results obtained for the type I phase were then compared with orientation studies of retinal in some lamellar liquid crystalline phases. These latter samples were macroscopically aligned between glass plates and the aligned lamellae were then studied in the light spectrometer at an inclined incidence of linear polarized light as illustrated in Figure 6 (cf. also Ref. 17 and 18). The dichroic ratio, D_ω , for a lamellar sample, having the angle ω between the direction of the propagation of light and the normal to the lamellae is given by the equation

$$D_\omega = \frac{A_\omega}{A_\perp} = 1 + 3S(1 - S)^{-1}n^{-2}\sin^2\omega \quad (4)$$

where S is the order parameter and n is the refractive index of the lamellar phase. A_ω and $A_\perp$ are the measured absorbances with the electric field

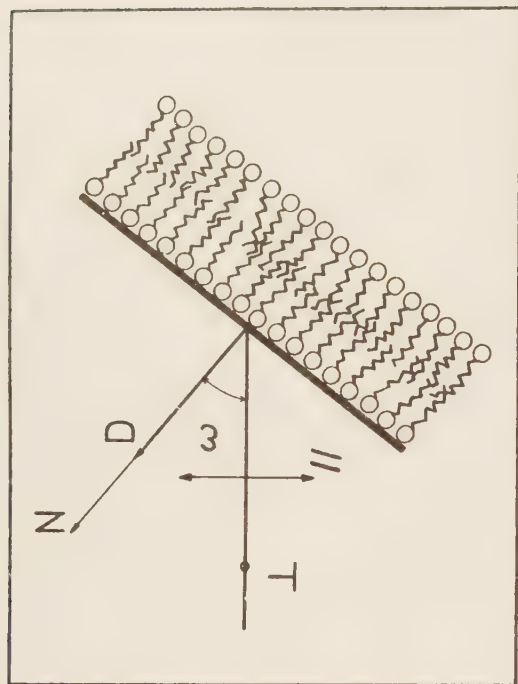


FIGURE 6 Schematic picture showing the polarizations and the direction of propagation of the incident light with respect to the normal N of the plates. The normal coincides with the director, D , of the lamellae.

vector of light forming the angles ω and $\pi/2$ relative to the director, respectively. For a lamellar sample composed of 16.94% w/w sodium octanoate, 35.77% w/w decanol, 47.29% w/w water and spurious amounts of retinal we obtained $S = 0.41 \pm 0.02$ ($n = 1.43$). Samples containing sodiumoctyl sulphate, decanol and water gave order parameters of similar values. The dependence of S on the composition was found to be small at these high water contents (Johansson, L. B.-Å., Söderman, O., Fontell, K. and Lindblom, G., to be published.) Thus the retinal molecule is highly oriented in these lamellar phases, probably due to its aldehyde group which is more or less "anchored" at the polar-nonpolar interface of the lamellae.

Figure 7 summarizes the data obtained from polarized absorption studies on retinal solubilized in small amounts in a type I sample oriented in a magnetic field, **B**. Two different field strengths have been used, namely 2.3 T and 6 T. For the latter field strength a superconducting magnet was used, where the sample tube axis is along the magnetic field instead of perpendicular to it as for 2.3 T, and therefore the orientation of the director of the sample is perpendicular to each other in the two cases. The order parameter is related to the measured absorbances, $A_\parallel$ (parallel to the director) and $A_\perp$ (perpendicular to the director) through the dichroic ratio, ψ (i.e. $\omega = 90^\circ$ in Eq. 4)

$$D = \frac{A_\parallel}{A_\perp} = \frac{1 + 2S}{1 - S} \quad (5)$$

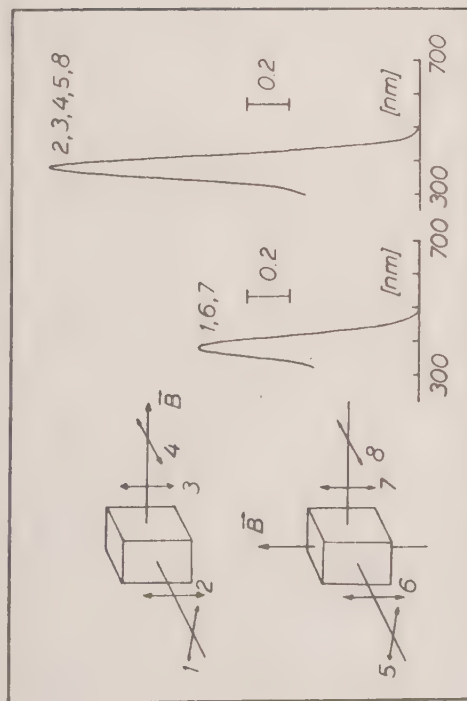


FIGURE 7 Absorbance of retinal solubilized in a type I sample measured with different directions of the linear polarization relative to the magnetic field vector, **B**. Two different magnetic field strengths have been utilized namely 2.3 T and 6 T, represented by the upper left and the lower left drawings, respectively.

The order parameter obtained in the experiments at both magnetic fields was found to be equal to $-0.17 (\pm 0.02)$. It should also be noted, as can be seen in Figure 7, that the absorbance, when the incident light is along the magnetic field, i.e. along the director, is independent of the polarization direction.

Spectroscopic,^{12,19} NMR diffusion¹⁰ and thermodynamic²⁰ observations strongly indicate that the local environment for an amphiphilic molecule is the same in a rodlike and a lamellar aggregate. Assuming that this is true also for retinal, the order parameters for the sample with rodlike aggregates is $-\frac{1}{2}$ of that for the lamellar liquid crystalline sample. We obtain -0.17 for the type I phase as compared to 0.41 for a lamellar sample. This, together with our NMR data, strongly indicate that the type I phase structure consists of rodlike aggregates.

NMR diffusion studies

We have also investigated whether the rodlike aggregates of the type I phase extend only over a small distance or if they have lengths of macroscopic distances. In previous works,^{9,10,15} we have shown that such problems are best tackled by studying the amphiphile translational diffusion using pulsed NMR with pulsed magnetic field gradients. The diffusion coefficient along the rodlike aggregates was determined for a macroscopically aligned sample in a magnetic field. The aligned sample was oriented in the magnet so that the angle between the director and the

magnetic field was equal to the "magic angle", 54.7° . By applying magnetic field gradients over the sample the diffusion coefficient was obtained from the spin echo as described in the experimental section. The amphiphile diffusion coefficient along the rods was determined with this method and was found to be $D_L = 8.7 \cdot 10^{-11} \text{ m}^2/\text{s}$. This value compares well with the lateral diffusion coefficient previously determined for octylsulphate in a lamellar mesophase.⁹ Since no restricted diffusion is observed it can be concluded that the rods must be quite long and by using, $\langle x^2 \rangle = 2Dt$, the length of the rods can be estimated to be longer than 2000 nm .

CONCLUDING REMARKS

Taken together, all our findings strongly indicate that the type I phase consists of long rodlike aggregates (in agreement with the location in the phase diagram). These rods orient themselves along the magnetic field direction (or, when glass plates are used, randomly in the plane of the glass plates). It is hard to conceive of any other structure that will explain all our experimental results. Our preliminary results suggests that the type II structure consists of lamellar aggregates.

Finally, it should be noted that magnetically orientable lyotropic mesophases are very suitable to use as matrices for studies of oriented polar molecules not only with NMR but also with polarized light spectroscopy methods.

Acknowledgements

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VII

A MAGNETIC FIELD ORIENTABLE LYOTROPIC LIQUID CRYSTAL.
AN ORDERING MATRIX IN LIGHT SPECTROSCOPY.

by

Lennart B.-Å. Johansson, Olle Söderman, Krister Fontell
and Göran Lindblom

Division of Physical Chemistry 2
Chemical Centre
University of Lund
S-220 07 Lund 7
Sweden

Introduction

Mixtures of amphiphiles and water (with or without salts) may form anisotropic fluids that align spontaneously in a strong magnetic field¹ i.e they behave as ordinary thermotropic nematics. They also may exhibit thread-like textures and the microscopic texture^{2,3} looks "nematic", and consequently they are often called lyotropic nematics. These systems are frequently being used as ordering matrices for NMR studies of the structure of dissolved molecules⁴. Compared to thermotropic phases, these phases have a great advantage since both hydrophobic and hydrophilic solutes can be dissolved.

Several investigations, mainly with NMR methods, of the physico-chemical properties of lyotropic nematic phases have been reported. Important contributions in this field are studies of the thermodynamic stability⁵ and determinations of the aggregate structure building up these phases⁵⁻⁸. In our structural study⁸ we utilized several spectroscopic techniques such as deuterium NMR, pulsed NMR for diffusion studies and polarized absorption spectroscopy. It was also suggested that these phases should be very suitable for photo-physical studies of chromophores with polarized light spectroscopy, both absorption and emission. One important application of these ordering matrices is their use in determinations of the electric transition dipole moments in chromophores^{9,10}. A prerequisite for a good optical ordering matrix is that the matrix itself does not absorb light in the wavelength region of interest, usually between 200-800 nm. The lyotropic nematic, discovered by Lawson and Flautt¹ containing the amphiphile sodium decylsulphate, absorb up to about 350 nm which unfortunately makes this system less suitable for many chromophores. Therefore, in this communication we have studied the optical properties of another lyotropic nematic discovered by Long and Goldstein¹². This system contain the amphiphile potassium laurate, permitting absorption studies of chromophores at lower wavelengths than the decylsulphate system. Using the same method as in our previous work⁸ we have studied the structure of the potassium laurate nematic. The time dependence of the macroscopical alignment of the system after switching off the magnetic field has also been investigated.

Experimental Section

Sample preparation. The nematic used had the composition 34.0 wt-% potassium laurate dodecanoate, 2.25 wt-% potassium chloride and 63.75 wt-% heavy water. The composition and the method of preparation of the mesophases is similar to that described previously¹¹.

²H NMR studies. Spectra were recorded at 15.351 MHz on a Varian XL-100 NMR spectrometer operating in the FT mode as described in previous work⁸.

NMR diffusion measurements. The amphiphile diffusion studies were performed on a Bruker 322 s pulsed NMR spectrometer equipped with a homebuilt pulsed magnetic field gradient unit as described previously¹³. For the determinations of diffusion coefficients of amphiphiles in lyotropic liquid crystalline phases macroscopically aligned samples were utilized^{8,13-15}. For experimental details see previous work^{13,14}.

Polarized absorption studies. The absorption spectra were recorded on a Varian spectrometer, Cary 219 supplemented with sheet polarizers (HNP'B, Polarizers, Ltd.). The samples were kept in a magnetic field for at least 12 h before the measurements of the absorbance was started. The absorption spectra were obtained with linear polarizers parallel ($A_{||}$) and perpendicular ($A_{\perp}$) to the magnetic field. The chromophores studied were retinal (Sigma), 2,2'-diethylthiocarbocyanide iodide (synthesized at "Syntestjänst, Lund"), ethidium-bromide (Sigma), methylorange (Merck) and retinal (Sigma). The spectra were recorded at 22°C.

Results and Discussion

Information about the structure of the nematic phase can be extracted from a combination of different spectroscopic investigations⁸. The orientation of the director for samples macroscopically aligned between glass plates determines the shape of the deuterium NMR spectra and an indication of

the amphiphile aggregate geometry can be obtained. A comparison of the molecular orientation relative to the director of a solubilized amphiphilic chromophore, like retinal in the nematic phase, with the orientation of the chromophore in a known lyotropic structure may give strong support for a certain aggregate shape. Similarly, by using specifically deuterated amphiphilic molecules in deutron NMR, values of corresponding order parameters for the different systems can be obtained, but unfortunately with an unknown sign in contrast to results from polarized light spectroscopy. Therefore the latter method is advantageous here. Finally an estimate of the size of the aggregate building up the nematic phase can be obtained from NMR diffusion measurements. These spectroscopic methods have been used in a previous paper⁸ where the structure of a nematic phase containing sodium decylsulphate was investigated.

Studies of the phase structure. The alignment in a magnetic field was followed by observing the change with time in the water ^2H NMR spectra. A completely aligned sample gives rise to a spectrum consisting of two equally intense peaks, the separation of which defines the quadrupole splitting, $\Delta(\theta)$ given by

$$\Delta(\theta) = |\sum p_i v_Q^i S_i (3\cos^2\theta_{LD} - 1)| \quad (1)$$

where p_i is the fraction of deuterons in site i , v_Q^i is $3/4\chi$ (χ = quadrupole coupling constant), S_i is the order parameters of water molecules in site i and θ_{LD} is the angle between the z -axes of the laboratory and the director coordinate systems. Fig. 1 shows a ^2H NMR spectra typical for a fully oriented sample obtained after about 50 min in the magnetic field. A rotation 90° around the sample tube axis leads to a reduction of a factor 2 in the quadrupolar splitting. Thus according to equation 1 the director of the macroscopically aligned sample is parallel with the applied magnetic field, B_0 . For samples aligned between glass plates the NMR spectra shown in Fig. 2 were obtained at different angles between the glass plates and the magnetic field. The angle dependence observed in the NMR spectra could be simulated for a model with the director randomly oriented in a plane^{8,17,18} are also shown in Fig. 2. It can be seen

that the agreement between the experimental and simulated spectra is convincingly good, strongly indicating that the director is randomly oriented in the plane of the glass plates.

The shape of the amphiphile aggregates in lyotropic liquid crystalline phases can be investigated with polarized light spectroscopy^{8,10}. This is feasible from a determination of the order parameter of a chromophore in different liquid crystalline structures. The underlying assumption for such an approach is that the chromophore orientation in the various structures (known and unknown) is the same relative the surface normal of the aggregate^{14,18-20}. Thus the observed order parameters is entirely determined by the aggregate geometry. Then this parameter for a phase having rodlike aggregates is $-\frac{1}{2}$ of that for a lamellar one.

In these studies small amounts of retinal was solubilized in the nematic phases and also in some ordinary lamellar liquid crystalline phases, one with the composition 11.85 % sodium octanoate, 28.15 % decanol and 60 % water and another one composed of 15.01 % sodium decylsulphate, 20.02 % decanol and 64.97 % water. The order parameters obtained for these phases were equal to 0.41 (± 0.02) and 0.36 (± 0.02) respectively. The experimental details are given in refs. 8 and 10. For retinal solubilized in the nematic phases, with potassium laurate (See Fig. 3) or sodium decyl sulphate as the amphiphiles the order parameters were - 0.22 and - 0.17 (value from ref. 8), respectively. These values were obtained from a measurement of the dichroic ratio given by

$$D = \frac{A_{\parallel}}{A_{\perp}} = 1 + \frac{3S}{1-S} \quad (2)$$

where $A_{\perp}$ and $A_{\parallel}$ are the absorbances measured parallel and perpendicular to the director, respectively. A comparison of the data obtained for the lamellar phases and the nematic phases thus reveals that the order parameters differ by a factor -0.5 and this results together with the previous NMR data therefore strongly indicate that the phase structure is composed of rodlike aggregates. The amphiphilic molecules are restricted to move within the rodlike aggregates. By means of a pulsed NMR method this restricted translational diffusion can be studied and an estimate of the extension of the aggregates may be obtained. The amphiphile

diffusion coefficient along the rodlike aggregates was determined for a in a magnetic field macroscopically aligned sample. This sample was then oriented at the magic angle between the director and B_0 as described previously⁸. The following results were obtained for the two nematic systems studied: the lateral diffusion along the rod $D_L = 5.3 \cdot 10^{-11} \text{ m}^2 \text{ s}^{-1}$ for potassium laurate and $D_L = 8.7 \cdot 10^{-11} \text{ m}^2 \text{ s}^{-1}$ for sodium decyl sulphate. These values compares well with the lateral diffusion coefficients previously determined for corresponding lamellar mesophases. From this investigation it can be concluded, since no restricted diffusion was observed, that the aggregates must be quite long. By means of the relation $\langle x^2 \rangle = 2Dt$ their length can be estimated to be longer than about 2000 nm.

Combined all these experimental findings are compatible with a phase structure consisting of long rod-like aggregates. The rod orient in magnetic field with the symmetry axis parallel to the applied magnetic field.

The polarized absorption spectra of various chromophores solubilized in the lyotropic nematic are shown in Fig. 4 and the spectrum of the matrix itself in Fig. 5. As can be inferred from the latter figure the mesophase absorbs light strongly at wavelengths below about 200 nm i.e. dissolved chromophores cannot be studied in this region. The time dependence of the order parameter, S , of metylorange in an aligned sample, without an applied magnetic field, is shown in Fig. 6. The observation of a constant S during at least 100 h means that the randomization preceeds extremely slowly. Therefore this lyotropic nematic is a very convenient matrix for light spectroscopic studies since it also has a high transmission over a wide range of wave lengths.

Acknowledgements

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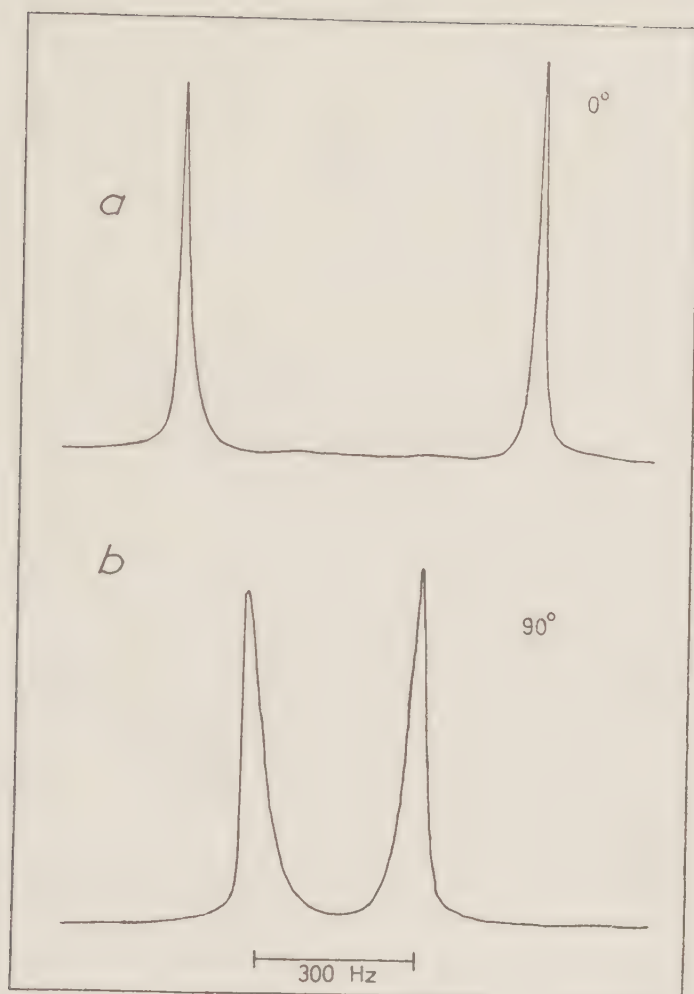


Fig. 1. ^2H NMR quadrupole splittings of $^2\text{H}_2\text{O}$ for the lyotropic nematic phase oriented with the director parallel (a) and perpendicular (b) to the magnetic field after a rotation of the oriented sample by 90° . The ratio between the splittings is equal to 2.

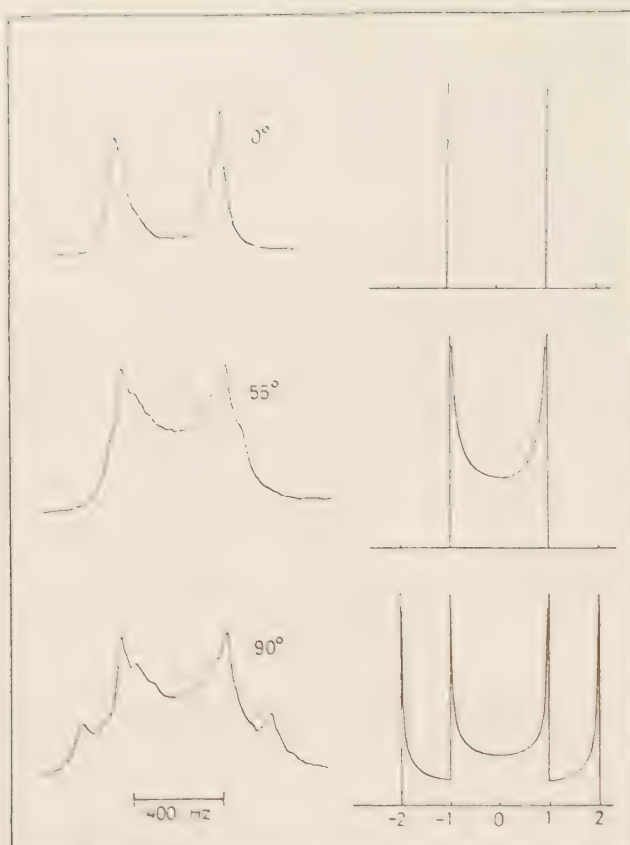


Fig. 2. ^2H NMR quadrupole splittings for the lyotropic nematic phase macroscopically aligned between glass plates at different approximate angles in the magnetic field are shown to the left. The simulated spectra for a sample having its directors isotropically distributed in the plane of the glass plates are drawn to the right. The frequency scale is in units of $4\nu/3\chi_S$. The angles correspond to that between the normal of the glass plate and the magnetic field.

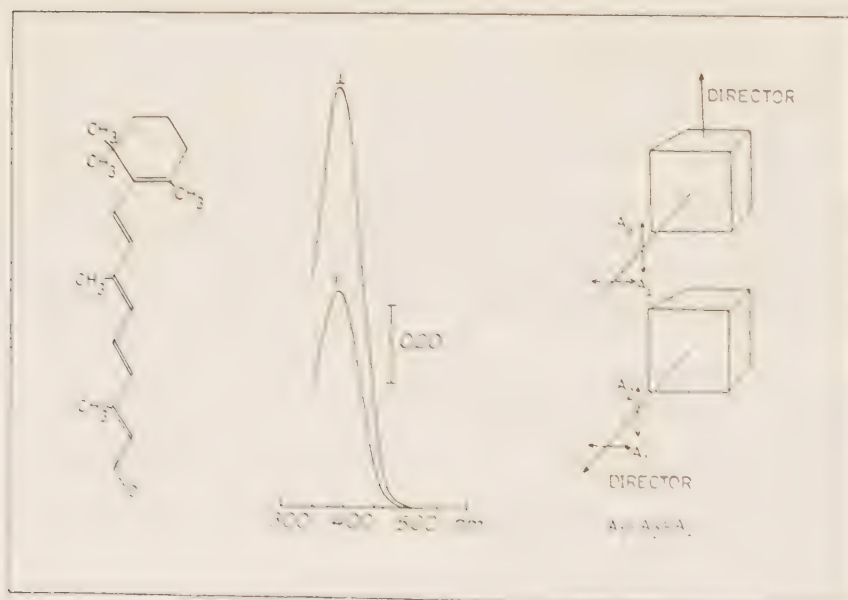


Fig. 3. Polarized absorbance spectra of retinal solubilized in the macroscopically aligned lyotropic nematic phase. The measurements are made for different directions of the polarizations relative to the director.

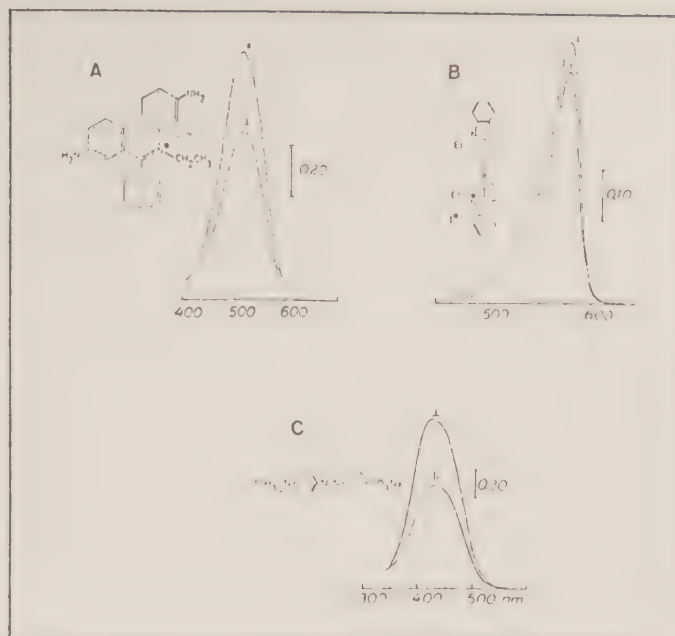


Fig. 4. Polarized absorption spectra of a) ethidiumbromide b) 2,2'-diethylthiocarbacyanide iodide c) methylorange solubilized in the macroscopically aligned lyotropic nematic phase.

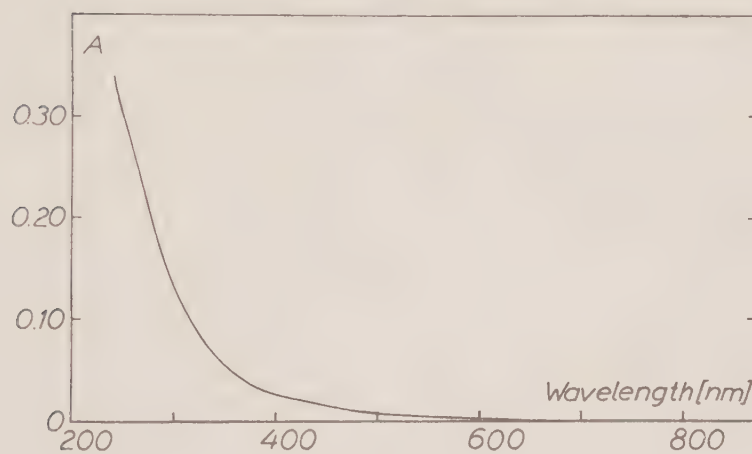


Fig. 5. Absorption spectrum of the lyotropic nematic matrix itself. Pathlength 1 cm.

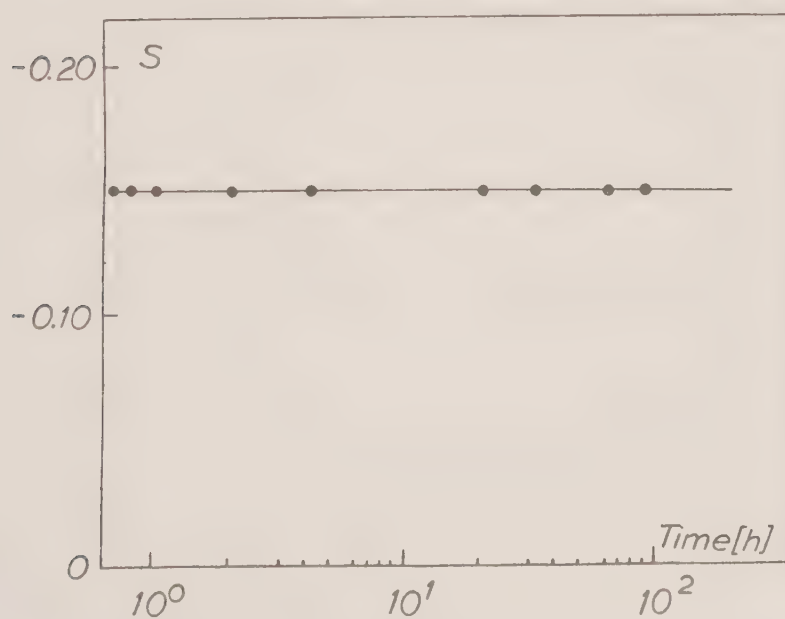


Fig. 6. Time dependence of the order parameter (S) of methylorange in the absence of magnetic field.

VIII

FLUORESCENCE DETECTED LINEAR DICHROISM

A new method for studies of molecular orientation in
uniaxial systems

by

Lennart B.-Å. Johansson, Göran Lindblom and *K. Razi Naqvi

Physical Chemistry 2, Chemical Centre
University of Lund
P.O.B. 740
S-220 07 LUND 7
Sweden

*Department of Physics, NLHT
University of Trondheim
N-7000 TRONDHEIM
Norway

Abstract

A theoretical and experimental description of a new light spectroscopic method for determination of second rank order parameters is carried out. The linear dichroism is obtained from measurements of a total emitted fluorescent intensity. This intensity is measured by using an integrating sphere. The method has been tested on a dichroic sample consisting of a stretched polymer film with an incorporated dye (2,2'-diethyl thiocarbonyl cyanide iodide). The main advantages of using emitted light is the high sensitivity and selectivity. With the method described the molecular orientation of a fluorophore in a mixture of other absorbing chromophores may be studied. It is also suggested that the integrating sphere should be used in determinations of excitation spectra and for measurements of fluorescence detected circular dichroism.

Introduction

Several different spectroscopic methods are being used for studies of molecular orientation in various anisotropic systems; crystals, liquid crystals, lipid bilayers, biological membranes and polymer films. The common techniques available are based on magnetic resonance, ESR¹ and NMR² or on polarized light absorption^{3,4} and emission^{4,5}, but also Raman spectroscopy⁶ can be utilized. Generally the light spectroscopic methods are more sensitive than the magnetic resonance ones but on the other hand the former are limited to systems containing chromophores. Furthermore the absorption and emission techniques usually requires macroscopically aligned samples. Therefore in studies of for example bilayers and membranes rather thin samples must be used. In addition, for a system containing several different chromophores it is desirable that the method is selective.

The experimental results obtained for uniaxial systems are usually interpreted in terms of order parameters introduced by Saupe². This parameter characterizes the average molecular orientation relative to the symmetry axis of the system, often called the director. For light spectroscopic methods this information can be extracted since the absorption of linear polarized light of an anisotropic sample will depend on the orientation of the sample in the light beam, i.e. the sample is (linearly) dichroic. This linear dichroism (LD) may be obtained experimentally by measurements of either the difference or the ratio of the absorbances with mutually perpendicular linear polarizations. The ratio so obtained is called the dichroic ratio. The order parameter is then conveniently calculated⁴ from the measured dichroism. A similar analysis of results from conventional fluorescence of an uniaxial system is much more complicated⁴ but give on the other hand, for an immobile fluorophore, more detailed information about the molecular orientation, since also fourth rank order parameters may be obtained. However, since fluorescence is quite sensitive and selective compared to absorption it would be advantageous to use emitted light for investigations of molecular orientation of samples having low chromophore content, which is often the case for biological systems. In this work such a method is described, based on measurements of the total emitted fluorescence intensity from which the second rank order parameters can be easily obtained.

Theory

The fluorescence intensity (F) emitted from an ensemble of molecules can be described by⁴

$$F = k \mathbf{T}^L \langle \mathbf{F}^L \rangle \mathbf{T}^{L,t} \quad (1)$$

Here k is a constant and $\langle \mathbf{F}^L \rangle$ denotes a 3×3 matrix representation of the emission polarization tensor averaged over an orientational distribution (isotropic or anisotropic). $\mathbf{T}^L$, a 3×1 matrix, represents the transmission axis of the analyzer (i.e. a linear polarizer). The superfix, L , indicates that the matrix elements are given in a laboratory coordinate system and t stands for the transpose. The fluorescence experiment can be performed in different ways, but here we will choose the polarization of the exciting electric field vector to be either perpendicular ($\perp$) or making an angle ω (see Fig. 1) with the laboratory Z -axis (the director). The ensemble mean values of the emission tensor are

$$\langle \mathbf{F}_\omega \rangle = \langle \mathbf{A}_\omega \mathbf{Q}^L \rangle \quad (3)$$

$$\langle \mathbf{F}_\perp \rangle = \langle \mathbf{A}_\perp \mathbf{Q}^L \rangle \quad (4)$$

where $\mathbf{A}_{\omega,\perp}$ are the appropriate absorbances. The matrix elements of the emission tensors are given by⁴

$$\begin{aligned} \langle F_{ij} \rangle_\omega = \int_{\Omega_0 \Omega} [P_{XX}^L(\Omega_0) \sin^2 \omega + P_{ZZ}^L(\Omega_0) \cos^2 \omega - P_{XZ}^L(\Omega_0) \sin 2\omega] Q_{ij}^L(\Omega) f(\Omega_0) g(\Omega_0 | \Omega, t) \times \\ \times w(\Omega, t) d\Omega_0 d\Omega \end{aligned} \quad (5)$$

$$\langle F_{ij} \rangle_\perp = \int_{\Omega_0 \Omega} P_{YY}^L(\Omega_0) Q_{ij}^L(\Omega) f(\Omega_0) g(\Omega_0 | \Omega, t) w(\Omega, t) d\Omega_0 d\Omega \quad (6)$$

P_{ij}^L and Q_{ij}^L are elements of the absorptivity tensor $\tilde{\mathbf{P}} = \vec{\mathbf{p}} \otimes \vec{\mathbf{p}}$ and the emission transition moment tensor $\tilde{\mathbf{Q}} = \vec{\mathbf{q}} \otimes \vec{\mathbf{q}}$, respectively $\vec{\mathbf{p}}$ and $\vec{\mathbf{q}}$ are transition dipole moments. $g(\Omega_0 | \Omega, t)$ is the conditional probability

density that if at a time zero a photon is absorbed by the molecule with the orientation Ω_0 then at a time t the emitting molecule is at Ω ($\equiv \alpha, \beta, \gamma$, the eulerian angles). Both molecular motion such as rotational diffusion as well as energy migration between molecules are described by g . The distribution density function of the fluorophores is $f(\Omega_0)$. Further, $w(\Omega, t)$ describes the probability of emission at time t for a molecule having the orientation Ω . Since the trace of $\langle F \rangle$ is invariant under rotation of coordinate system the Ω dependence of $\langle F \rangle$ will vanish and $\text{Tr} \langle F \rangle$ equals q^2 . Thus the information of the molecular orientation is solely confined to the absorption tensor element P_{ij}^L .

Hence

$$\text{Tr} \langle F_{\omega} \rangle = \int_{\Omega_0} \int_{\Omega} [P_{XX}^L(\Omega_0) \sin^2 \omega + P_{ZZ}^L(\Omega_0) \cos^2 \omega - P_{XZ}^L(\Omega_0) \sin 2\omega] q^2 f(\Omega_0) g(\Omega_0 | \Omega, t) w(\Omega, t) d\Omega_0 d\Omega \quad (7)$$

$$\text{Tr} \langle F_{\perp} \rangle = \int_{\Omega_0} P_{YY}^L(\Omega_0) q^2 f(\Omega_0) g(\Omega_0 | \Omega, t) w(\Omega, t) d\Omega_0 d\Omega \quad (8)$$

Now since $\int_{\Omega} g(\Omega_0 | \Omega, t) d\Omega = 1$ and assuming that w is independent of Ω lead to that Eqs. 7 and 8 can be written

$$\text{Tr} \langle F_{\omega} \rangle = q^2 w(t) \int_{\Omega_0} [P_{XX}^L(\Omega_0) \sin^2 \omega + P_{ZZ}^L(\Omega_0) \cos^2 \omega - P_{XZ}^L(\Omega_0) \sin 2\omega] f(\Omega_0) d\Omega_0 \quad (9)$$

$$\text{Tr} \langle F_{\perp} \rangle = q^2 w(t) \int_{\Omega_0} P_{YY}^L(\Omega_0) f(\Omega_0) d\Omega_0 \quad (10)$$

Note that anisotropic motion of the fluorophores has no effect in Eqs. 9 and 10. For a uniaxial system Eqs. 9 and 10 become (cf. ref. 4)

$$\text{Tr} \langle F_{\omega} \rangle = q^2 w(t) \left(\frac{1}{3} \text{Tr} \tilde{P} + \sum_{n=-2}^2 (\cos^2 \omega - \frac{1}{3}) \langle D_{on}^{(2)*}(\beta\gamma) \rangle P_n^M \right) = q^2 w(t) A_{\omega} \quad (11)$$

$$\text{Tr} \langle F_{\perp} \rangle = q^2 w(t) \left(\frac{1}{3} \text{Tr} \tilde{P} - \sum_{n=-2}^2 \langle D_{on}^{(2)*}(\beta\gamma) \rangle P_n^M \right) = q^2 w(t) A_{\perp} \quad (12)$$

$\langle D_{on}^{(2)*}(\beta, \gamma) \rangle$ are space averaged Wigner rotation matrix elements and P_n^M are the five irreducible components of the tensor $\tilde{P}$ expressed in the molecular coordinate system (M). It is obvious from Eqs. 11 and 12 that from appropriate measurements of the traces of the emission tensor the $\langle D_{on}^{(2)*} \rangle$ -elements are proportional to the order parameters introduced by Saupe² (see also ref. 4). Usually, measurements are performed by rotating the analyzer into mutually perpendicular positions. Three different mutually perpendicular settings are possible and any such polarizer settings $k=1,2$ and 3 can be described by

$$T_k^L = (\delta_{1k} \ \delta_{2k} \ \delta_{3k}) R(\theta, \rho) \quad (13)$$

where R is a cartesian rotation matrix⁷ and δ is the Kronecker delta. For a setting k

$$T_k^L \langle F \rangle T_k^{L,t} = \sum_{i,j} \langle F_{ij} \rangle R_{ik} R_{jk}$$

and

$$\sum_{k=1}^3 T_k^L \langle F \rangle T_k^{L,t} = \sum_{ijk} \langle F_{ij} \rangle R_{ik} R_{jk} = \sum_{ij} \langle F_{ij} \rangle \delta_{ij} = \sum_j \langle F_{jj} \rangle = \text{Tr} \langle F \rangle \quad (14)$$

Thus measurements of the fluorescence intensity in any three perpendicular settings of the analyzer give $\text{Tr} \langle F \rangle$, i.e. the absorbance A . This further implies that the total intensity emitted or any fluorescent intensity measured proportional to it will also yield $\text{Tr} \langle F \rangle$. This result constitutes the corner-stone in the method of fluorescence detected linear dichroism (FDLD) to be discussed in the next section.

Method

For isotropic solutions of low viscosity the fluorescence excitation spectrum may mimic the absorption spectrum⁸. Usually the emission is observed in only one direction, which is perpendicular to the propagation direction of light. For an anisotropic sample, however, the total intensity must be measured in order to get the absorption spectrum (cf. theory section). In order to quantify the molecular information several different experimental parameters can be defined and in the FDL method we introduce the emission ratio, s , as follows

$$s = \frac{\mathcal{F}_\omega}{\mathcal{F}_\perp} \quad (15)$$

Here, $\mathcal{F}_\omega$ and $\mathcal{F}_\perp$ have the meaning of the total emission intensity or a quantity proportional to it as was emphasized in the theory section. By definition the fluorescence intensity is related to the absorbed light intensity, I_a , through the quantum yield of fluorescence, ϕ by

$$\mathcal{F} = \phi I_a \quad (16)$$

I_a can be displaced by the absorption, A , in equation (16) by using $I_a = I_0(1 - e^{-A \ln 10}) \approx I_0 A \cdot \ln 10$ for optically dilute samples (small absorption), leading to

$$\mathcal{F} = \phi I_0 A \ln 10 \quad (17)$$

As can be seen from equations (7) and (8) the quantum yield of fluorescence may depend through $w(\Omega, t)$ on the anisotropy of the system studied. In the following we will, however, assume that ϕ is independent on the anisotropy. The treatment of an anisotropic dependent $w(\Omega, t)$ is very complicated and will be left for future investigations. Then equation (15) can be written

$$s = \frac{\mathcal{F}_\omega}{\mathcal{F}_\perp} = \frac{A_\omega}{A_\perp} = \frac{F_\omega}{F_\perp} \quad (18)$$

From the equations (11) and (12) with equation (18) a relation between the measured emission ratio s and the molecular orientation in the sample can be obtained

$$s = 1 + \frac{3 \sum_n \langle D_{on}^{(2)*}(\beta\gamma) \rangle P_n^M \cos^2 \omega}{\text{TrP} - \sum_n \langle D_{on}^{(2)*}(\beta\gamma) \rangle P_n^M} \quad (19)$$

Considering a molecule having only one transition moment with the molecular z -axis parallel to the transition dipole, n equals zero and

$$s = 1 + \frac{3S \cos^2 \omega}{1 - S} \quad (20)$$

where $S = \langle D_{00}^{(2)*} \rangle$ is the order parameter.

We will now consider a slightly more complicated system composed of two different kinds of molecules; one of which is nonfluorescent but absorbs light with the absorbance A and the other ones emit fluorescent light having the absorbance A^* . The measured fluorescence intensity will then be

$$\mathcal{F} = \phi \frac{A^*}{A + A^*} I_a \quad (21)$$

In analogy to equation (18) the emission ratio can be written

$$s = \frac{A_{\omega}^*}{A_{\perp}^*} \cdot R \quad (22)$$

where

$$R = \frac{A_{\perp}^t (1 - 10^{-A_{\omega}^t})}{A_{\omega}^t (1 - 10^{-A_{\perp}^t})} \quad (23)$$

with $A^t = A + A^*$.

The total absorbances for both the molecules A_{ω}^t and $A_{\perp}^t$ can be determined from ordinary polarized absorption measurements. The order parameters for the fluorescent molecules is then obtained from the ratio $\frac{A_{\omega}^*}{A_{\perp}^*}$ (cf. eq. 22),

which is equal to the right hand side of equation (19). Note that if

A^t is small (≤ 0.01) $R \approx 1$ and the dichroic ratio is entirely given from the fluorescence experiment. This shows the property of selectivity in the FDLD experiment.

In practice there are mainly two ways to obtain the total intensities $\mathcal{F}_{\omega}$ and $\mathcal{F}_{\perp}$. One possibility is to measure the fluorescence intensities in three mutually perpendicular directions (cf. equation 14).

The sum of appropriate intensities are directly proportional to $\mathcal{F}_{\omega}$ and $\mathcal{F}_{\perp}$. This method is tedious and time-consuming. A more convenient and accurate technique is to utilize an integrating sphere⁹, with which the total fluorescence emission can be collected in a single measurement. Such a sphere (Figure 2) consists of a spherical cavity lined with barium sulfate, a highly scattering and nonabsorbing surface. A sample placed in the centre of the sphere will therefore emit polarized light which is scattered and depolarized at the wall of the cavity so that a total intensity can be detected. The experiments in this investigation have been performed with such an integrating sphere (Figure 3).

Results and Discussion

The FDLD method has been used to study the molecular orientation for the dye 2,2'-diethylthiocarbocyanide iodide incorporated in a stretched film consisting of polyvinylalcohol. Polarized absorption spectra of this sample are shown in Figure 4. The dichroic ratio $D = \frac{A_{\omega}}{A_{\perp}}$ was found to be equal to 6.7 at 570 nm corresponding to an order parameter $S = 0.69$. The directions of polarizations $\parallel$ and $\perp$ are relative to the director. Thus the large, positive S -value strongly indicate that the long axis of the chromophore tends to be oriented along the axis of stretching. It is assumed that the transition dipole moment is parallel with the long axis in the chromophore.

The emission ratio, s , has been determined in the wave length region 520 - 585 nm with the polarizations of the exciting light parallel and perpendicular to the axis of stretching. The data obtained are summarized in Figure 5 together with the emission ratio calculated from the polarized absorption spectra (cf. Eqs 22-24). It can be inferred from this figure

that there is a good agreement between the emission and absorption results carried out on the same sample. It should be noted, however, that these experiments are considered to be a first check only of the FDLD-method and no attempt has been made yet to get a better accuracy. Future investigations are subjected to improvements of the technique.

Compared with ordinary absorption methods there are mainly two great advantages to use FDLD for studies of molecular orientation of uniaxial systems. First, much lower concentrations of the chromophore can be used i.e. very thin samples can be studied. Therefore FDLD will be more sensitive than polarized absorption. Second, for samples containing different chromophores having overlapping absorption spectra, but where only one of these chromophores is fluorescent, the dichroic ratio of this molecule may be conveniently extracted. Thus FDLD will have a selectivity that the absorption experiment is lacking. Both these properties inherent in the FDLD method are of outmost importance in the study of the complex biological systems as for example biological membranes. We therefore believe that FDLD will have its greatest merits in studies of various macroscopically aligned membrane systems.

Finally it must be noticed that the use of an integrating sphere is highly advantageous in ordinary fluorescence⁸ and fluorescence detected circularly dichroism¹⁰ of viscous systems. The reason for this is that the excited molecules exhibit anisotropic emission in a system of high viscosity. This is due to the fact that the brownian rotational motion will be insufficient in making the space average isotropic within the fluorescence lifetime. The integrating sphere, on the other hand, removes this anisotropic property of the system by being an effective depolarizer of the emitted light.

Experimental

The polyvinylalcohol (PVA) films were prepared as described by Davidsson and Nordén¹¹. PVA (elvanol 71-30) was bought from DuPont Nemours Ltd. and 2,2'-Diethylthiocarbacyanide iodide was synthesized at "Syntestjänst Lund". A nitrogen laser (NRG-0.7-5-200, National Research Group, Inc.) pumping a dye laser (Spectrolas 3B, Jobin Yvon) was used as light source. The fluorescence from the sample was passed through a long pass filter (G-772-6300, Oriel) and detected by a red sensitive photomultiplier (R 928, Hamamatsu), the output of which was averaged by boxcar detector (9415/9425, Brookdeal). The integrating sphere (Cf. Figure 3) was made of aluminium. The diameter of the spherical cavity is 170 mm and the inside wall lined with BaSO₄ (Merck). Absorption spectra were recorded on a Varian Cary 219 spectrophotometer. The fluorescence experiments were performed at the Department of Physics at the University of Trondheim.

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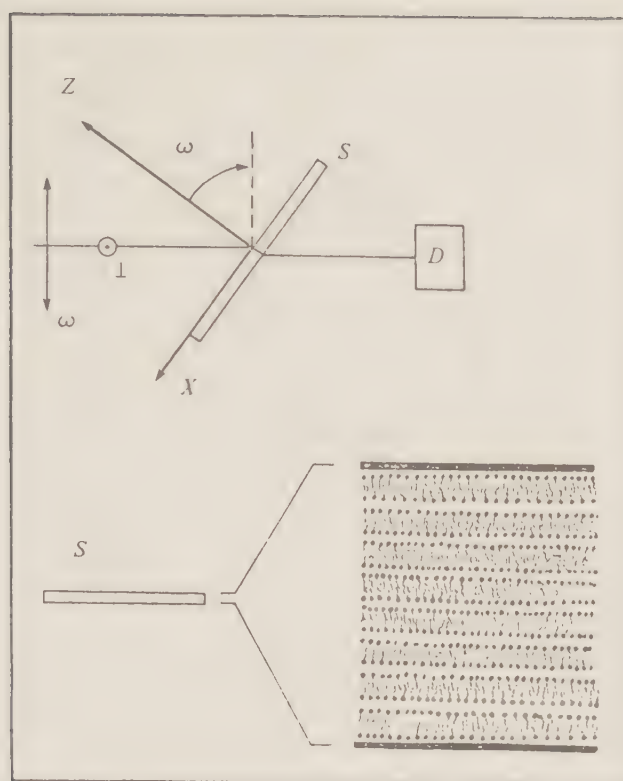


Figure 1. Excitation with the linear polarizations perpendicular (**1**) and at an angle ω relative to the director. The laboratory Z axis is chosen parallel to the director. S, the macroscopically aligned sample could e.g. be a lamellar liquid crystalline phase as illustrated here.

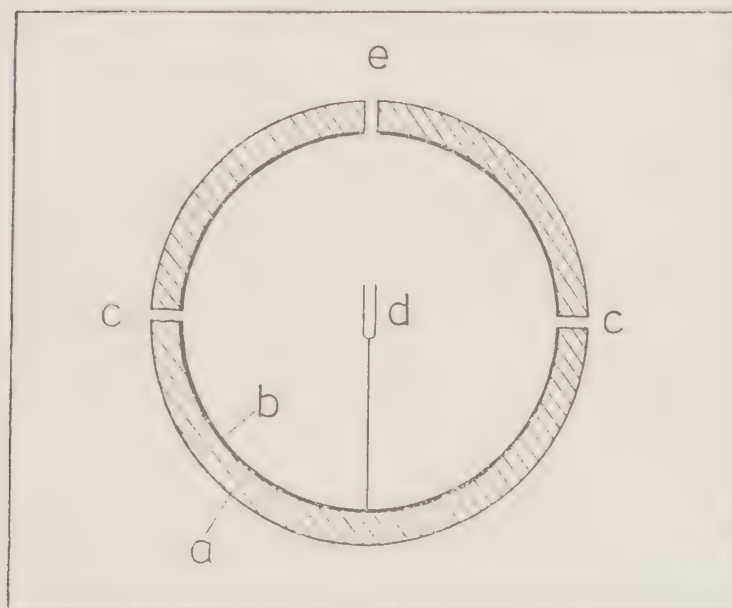


Figure 2. Integrating sphere.

- a) spherical cavity made of aluminium (inner diameter 170 mm)
- b) scattering layer of barium sulphate (thickness ≈ 1 mm)
- c) in- and out-let holes for the excitation light beam (diameter 5 mm)
- d) sample holder made of glass or quartz
- e) out-let hole for emitted light.

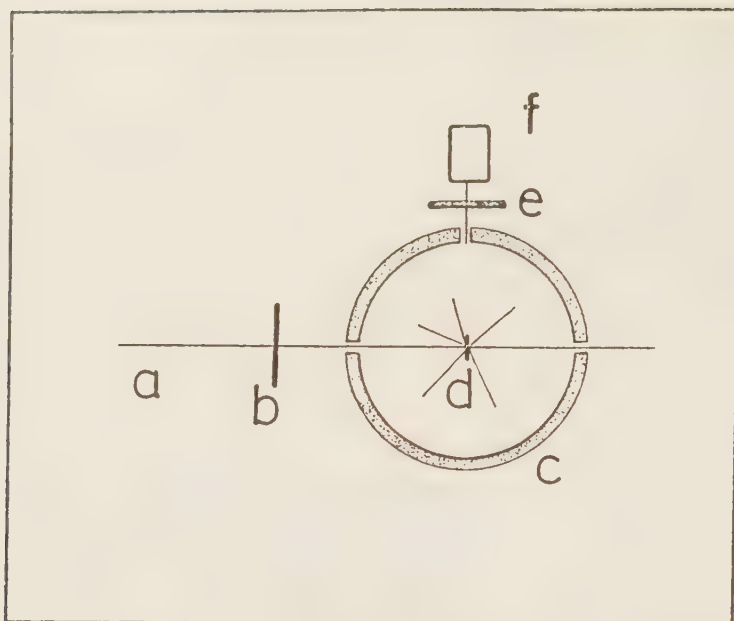


Figure 3. Schematic picture of the FLD apparatuses utilizing an integrating sphere.

- a) monochromatic excitation light
- b) linear polarizer
- c) integrating sphere
- d) fluorescent dichroic sample
- e) cut-off filter
- f) photomultiplier

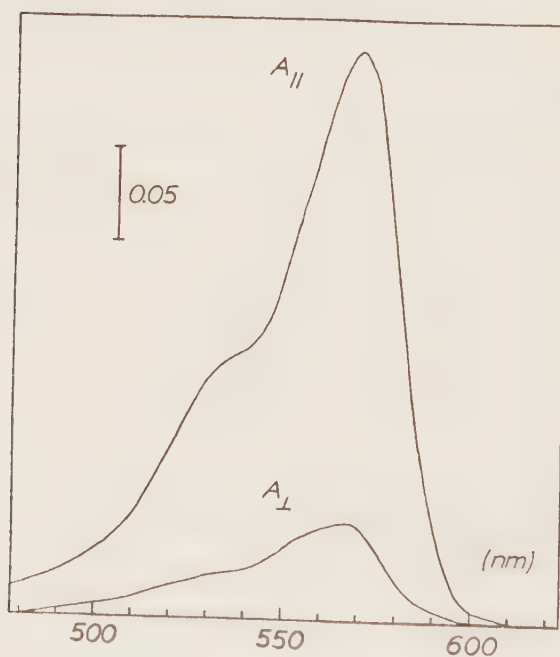


Figure 4. Polarized absorption spectra of 2,2'-diethylthiocarbonyl iodide incorporated in a stretched polyvinylalcohol film. $A_{||}$ and $A_{\perp}$ are the absorbances recorded with the polarizer set parallel and perpendicular to the stretching axis of the film (the director).

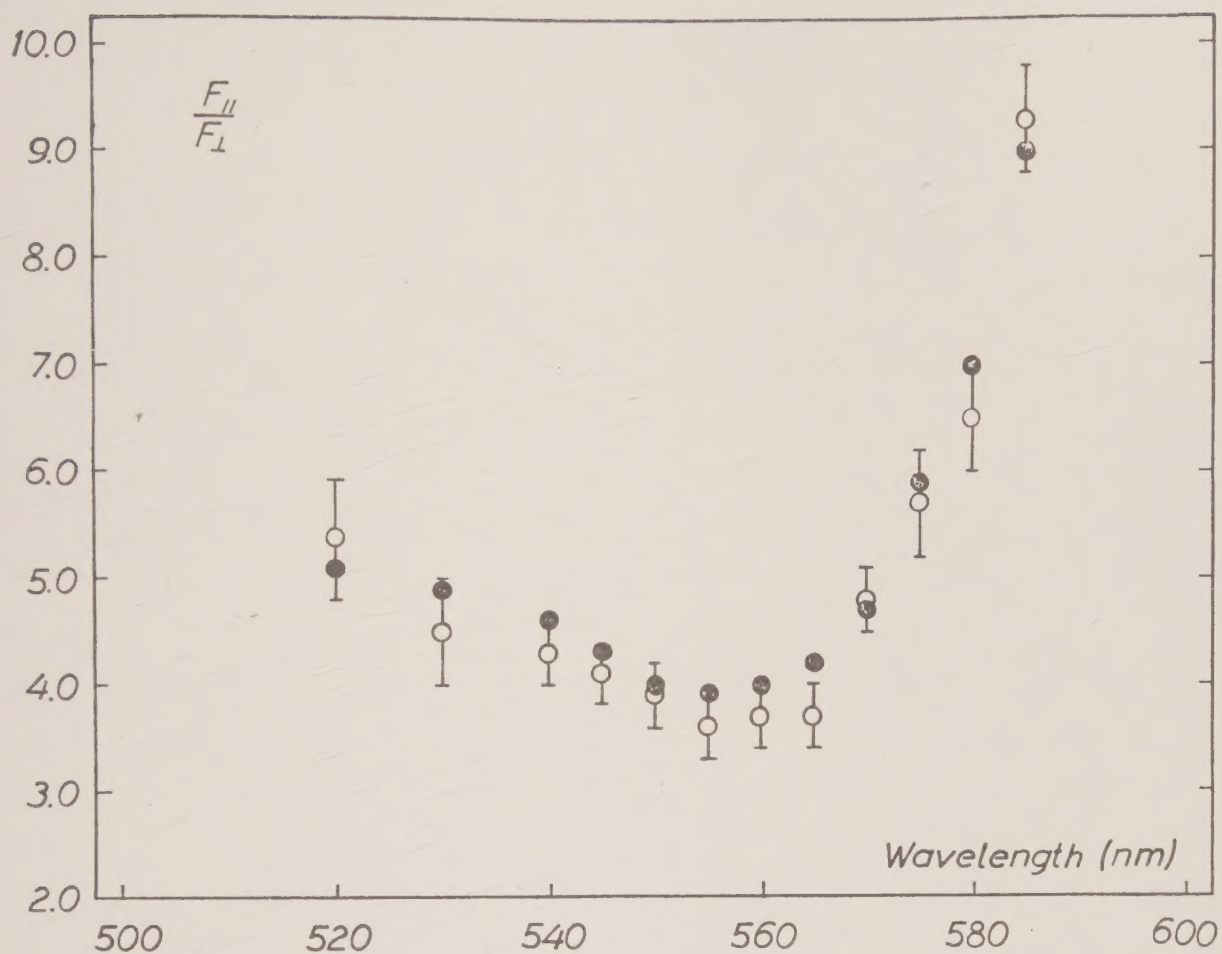


Figure 5. The emission ratios, $s = \frac{F_{||}}{F_{\perp}}$, of 2,2'-diethylthiocarbocyanide iodide determined from FLD measurements ($\circ$) and calculated from the polarized absorption measurements using equations 21-23, ($\bullet$).

